

**Adolescent Methylone Exposure and its Effects on
Behavioural Development in Adulthood**

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Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
ANOVA	Analysis of Variance
APA	American Psychological Association
CNS	Central Nervous System
dB	Decibel
DA	Dopamine
DSM-IV-TR	The Diagnostic and Statistical Manual of Mental Disorders
i.p.	Intraperitoneal
GAD	Generalised Anxiety Disorder
LSD	Lysergic acid diethylamide
Mg	Milligrams
mg/kg	Milligrams per kilogram
MDMA	Methylenedioxymethamphetamine (Ecstasy)
mm	Millimetre
ms	Millisecond
MPH	Methylphenidate (Ritalin)
MTH	Methylone
n	Number
NA	Noradrenalin
PND	Post Natal Day
S	Saline
SUD	Substance Use Disorders
VTA	Ventral Tegmental Area
$\alpha_4\beta_2$	Alpha-4 beta-2 nicotinic receptor
5-HT	Serotonin

Abstract

Originally developed as an anti-depressant and later available as a ‘party-pill’ in New Zealand, methylone is currently classed as an illegal drug. This is due to findings of its similarity in chemical structure to that of Ecstasy (MDMA). Methylone is a relatively new drug into which little research has been conducted. Consequently, no known study has investigated the long-term effects on behavioural development arising from exposure during adolescence. The present thesis therefore aimed to identify long-term effects of chronic adolescent exposure to methylone on adult anxiety-like behaviours. This was achieved by the use of 80 rats (40 males: 40 females) and exposing them to either a methylone or saline treatment for ten consecutive days. Two different treatment age groups (early versus late adolescence) were examined and to ensure adequate comparisons could be made, two control groups were utilised. All rats were tested during adulthood in four specifically selected anxiety-measure tests; the open-field, preference for the light side of a light-dark box, acoustic startle and responsiveness to the novel arm of a Y-maze. The results suggested methylone-exposed rats displayed more anxiolytic behaviours than saline-treated rats. In the open field methylone exposed rats exhibited less ambulation than controls and those treated in early adolescence defecated more while rats treated in late adolescence occupied the corners of the apparatus more exhibiting higher anxiety-like behaviours. Exploratory behaviours in the Y-maze were decreased in methylone-treated rats, and those exposed in early adolescence entered the novel arm less often. However, acoustic startle results suggested methylone-exposed rats were less anxious as evidenced by a lower startle amplitude than controls. Overall, the results suggested differences in anxiety-like behaviours between methylone-exposed rats and controls. It did not appear that being exposed to methylone in early adolescence resulted in vast differences in anxiety-like behaviours than if exposure began in late adolescence.

Introduction

1.1 General Overview

Adolescence is a vulnerable period in an individual's life encompassing the transition from childhood to adulthood (Spear, 2000). Decisions made within this developmental phase can positively or negatively impact upon future adult functioning. During adolescence, there is an increase in risk-taking behaviours, such as drug use, and individuals are highly influenced by peers (Arnett, 1996; Conner et al, 2010; Fowler et al, 2007). The use of substances in this essential phase of growth can be detrimental to future adult development and functioning (Young et al, 2006). Therefore, research needs to be undertaken to examine the developmental effects of taking substances during adolescence. This statement is especially relevant to new drugs where little research has examined their long term effects. As mentioned above, adolescents are prone to engaging in risky behaviours during adulthood and as such, are at risk for using a new substance of which little is known about.

Methylone is a recently discovered stimulant drug with a short history of human use (Shimizu et al, 2007). Methylone is similar to 3,4-methylenedioxymethamphetamine (MDMA) in its behavioural profile but differs structurally (Cozzi et al, 1999). Methylone is the benzylic ketone analogue of MDMA and contains an additional oxygen atom at the benzylic position of the molecule (Bossong, et al, 2005). Like MDMA, methylone acts on monoaminergic systems and results in a stimulation of the Central Nervous System (CNS) and hallucinogenic effects (Kamata et al, 2006). Due to the structural similarities between methylone and MDMA, health and developmental risks which are common to MDMA users would also be expected in users of methylone (Kamata et al, 2006). Unfortunately, literature and therefore, knowledge about the long-term effects of this new drug is sparse.

The use of illegal substances is increasing in New Zealand with one in two adults between the ages of 16 and 64 having tried an illegal drug in their life time (Ministry of Health, 2010). In 2007, the Ministry of Health found that MDMA was the third most popular recreational drug used in New Zealand and one in ten individuals aged between 18 and 24 years had used it in the last 12 months. Unfortunately, it is plausible that if methylone became readily available within New Zealand, its popularity could increase steadily due to similarities with MDMA and being seen as a safer alternative. Currently there is no known literature on the effects of using methylone during adolescence. It is the primary aim of this thesis to provide an assessment of the long-term behavioural effects in adulthood following adolescent exposure to methylone. In addition, this study would make an original contribution to scientific literature on the effects of methylone.

1.2 Substance Use/Abuse

Carlson (2007) explains addiction in a simple, understandable manner and states that the term ‘addictions’ is derived from the Latin meaning ‘to sentence’. That is, an individual who is addicted to a drug is, in a sense, sentenced to a term of unintentional servitude - they are obliged to fulfil the burden of a drug dependency (Carlson, 2007).

Drug addiction places a burden on societies all around the world, severely impacting on crime levels, health sectors, social cohesion and comorbidity with psychological disorders (Everitt et al, 2001; Shima, 2009). In order to fully understand the increasing phenomenon of substance abuse and addiction, a definition follows. The leading tool for diagnosing any mental disorder, including Substance Use Disorders (SUD), is the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). For a SUD diagnosis, a substance can be defined as anything which is ingested by an individual in order to produce a high, rush or to otherwise alter one’s affect functioning. For an individual to be diagnosed as an abuser or dependant,

they must first go through an experimental phase in which experimentation of the drug in question occurs (APA, 2000).

The DSM-IV-TR defines Substance Abuse as a pattern of substance use which leads to significant impact in an individual's functioning. To meet diagnosis, an individual must present with one of the four identified criteria within a 12-month period and additionally, must not simultaneously meet the criteria for Substance Addiction. Briefly, the four criteria that an individual must engage in are as follows: participate in substance use in situations that are potentially hazardous, such as while driving or operating heavy machinery; repeated use resulting in one's failure to complete essential obligations, such as work, school or at home; legal problems resulting from recurrent use; or continuing to use a substance despite social or interpersonal problems being caused by the substance (APA, 2000). Comparatively, the DSM-IV-TR states that for an individual to be substance dependant there is a pattern of repeated self-administration of the drug that can result in tolerance, withdrawal and compulsive drug-taking (APA, 2000). To meet the DSM's criteria for Substance Dependence, individuals must satisfy two sections. Firstly, an individual must present with three of seven identified symptoms and these symptoms must have been experienced at any time in the previous 12-month period to diagnosis (refer to APA, 2000 for a full description of these). Secondly, the individual must have never met the criteria for Substance Dependence for this class of substance. Individuals who do not meet the criteria for a substance dependant diagnosis, but who experience one of the symptoms are considered substance abusers.

The behavioural and subjective effects of a drug vary between individuals due to complex interactions between pharmacological, environmental and psychological factors (Cromberg & Robinson, 2004). For example, many soldiers in the Vietnam War who became dependant on Heroin stopped drug use once they returned to America and their homes (Cromberg & Robinson, 2004). This illustrates an environmental factor influencing drug use as once the soldiers left Vietnam (a triggering environment), they ceased the use of heroin.

1.2.1 *An Adolescent's Predisposition - Initiation of Drug Use*

Genetic, personality and environmental factors are variables which can contribute towards an individual's substance use and potentially giving them a predisposition to use drugs (Weinberg et al, 1998). These factors need to be explored to help determine why some adolescents choose to use illegal drugs while others' do not. Due to individual differences, the reasoning as to why some adolescents' initiate drug use is varied and complex and therefore, cannot be generalised to one predictable factor. Even so, there are several important environmental factors which can help contribute to a young person's initial drug taking. These include peer pressures, drug availability, financial means, and family influences (Cromberg & Robinson, 2004). As adolescents often experiment with drug use but have a lower tolerance threshold than adults, they are at a greater risk of becoming dependent at lower doses (Fowler et al, 2007). This when combined with any genetic predisposition of drug addiction, becomes a worrying factor for future drug dependence.

While it is not known exactly which factors cause drug abuse, it is recognized that drugs that lead to dependency must first reinforce one's behaviours (Carlson, 2007). Initially, the reinforcement needs to be positive so the behaviour (e.g. taking the drug) is immediately followed by a reward (e.g. feelings of confidence, energy, high self esteem, relief of stress). Therefore, the reward of the drug 'high' is reinforcing the behaviour of initially taking the substance. Generally for drugs of abuse, the mechanisms which support substance use are neurobiological.

A twin-study by Derringer et al (2008) analysed how genetic and environmental influences affected the total number of substances used during adolescence. Results found substance use increased over time for both sexes, and adolescent males reported a wider diversity of substances used than adolescent females. Interestingly, influences of genetic factors also increased with age. Genetic influence appeared to be greater for males. Correlations between genetic influences and substances used were maintained across ages

suggesting that genetic make-up is a significant risk factor in influencing adolescents' drug use.

1.2.2 Mechanisms of Drug Dependence

The use of many illegal drugs causes the release of dopamine within the mesocorticolimbic system- specifically the nucleus accumbens (Carlson, 2007; Carlezon & Konradi, 2004; Conner et al, 2010). This is often referred to as the 'reward' pathway as activation of this structure often leads to feelings of reward and pleasure (Bressan & Crippa, 2005; Everitt & Robbins, 2005). Reduced activity of this system can trigger drug seeking behaviours as individuals seek to reinstate a pleasurable state (Conner et al, 2010). It has been suggested that addiction occurs because drugs of abuse interfere with normal brain reward circuits that provide reinforcement for behaviours of survival such as food, water and sex (Niehaus et al, 2009). That is, the natural rewards gained from these survival behaviours are positively reinforced until the skill is learned. However, with a drug of abuse, the reward circuit is continually stimulated each time the drug is ingested. Consequentially, as positive reward reinforcement is required for both the learning of survival skills and also for a drug to be perceived as rewarding, this may suggest that the chronic use of drugs can shape behaviours.

The dopamine (DA) hypothesis suggests that most addictive substances exert their psychoactive actions on the neurotransmitter DA and the neural systems it regulates (Robbins & Everitt, 1999; Everitt & Robbins, 2005). The increase of DA levels within the mesolimbic DA system has been linked to the euphoric feelings and addictive properties of drugs (Carlson, 2007; Everitt & Robbins, 2005; Niehaus et al, 2009), as well as mediating several reinforcement events (McGregor & Roberts, 1993). It has been found in numerous studies that intravenous injections of cocaine or amphetamines increase the concentration of DA in the nucleus accumbens (Carlson, 2007). The nucleus accumbens is encompassed within the

Ventral Tegmental Area (VTA), a midbrain region that is a major site of DA neurons. It is in this brain region where the mesolimbic DA system is found (Niehaus et al, 2009).

The VTA has been shown to play a critical role in reinforcement behaviours. For example, when nicotine binds with $\alpha_4\beta_2$ receptors within the VTA, the DA pathway is stimulated. This leads to an increase in mesolimbic DA levels; this increase supplies the smoker with pleasurable feelings that reinforce the behaviour (Tsai et al, 2007; Rollema et al, 2007). If DA antagonists are injected into the nucleus accumbens, drugs such as cocaine and amphetamine lose much of their reinforcing effects (Carlson, 2007). This was illustrated in a study by McGregor and Roberts (1993) using trained rats to self-administer cocaine. The rats were then injected with a DA receptor antagonist into either the amygdala or the nucleus accumbens; this resulted in a significantly lesser quantity of cocaine being self-administered by the animals. When injected into the amygdala, the DA antagonist did not have any effect on the amount of cocaine self-administered. However, when the antagonist was injected into the nucleus accumbens, self-administration of cocaine was significantly reduced. This study suggests that if DA is blocked, certain drugs may lose their reinforcing effects as they are unable to interfere with the DA 'reward' circuit in the brain.

Recent studies have challenged the DA hypothesis, particularly those studying cocaine (Caine, 1998). Cocaine binds with and deactivates the DA transporter proteins, blocking the reuptake of DA once it is released by the terminal buttons (Carlson, 2007). This blockage leads to increased extracellular DA, it is this effect that is considered to be the prime cause of the reinforcing and addictive nature of cocaine (Caine, 1998). Research by Rocha et al (1998) found 'DA-transporter knockout mice' trained to self-administer cocaine still engaged in a pattern of behaviours in which the reinforcement of cocaine was found. This suggests that binding to the DA transporter is not required for cocaine reinforcement effects. Studies such as these place doubt on the validity of the DA hypothesis.

Although DA appears to be important for the reinforcement provided by drugs, research on serotonin (5-HT) is also persuasive. 5-HT is a neurotransmitter within the brain that contributes to feelings of well-being in an individual (Carlson, 2007). Therefore, when 5-HT is released the resulting positive feelings may cause a reinforcing effect of the drug taken. However, excessive 5-HT stimulation can lead to the 5-HT Syndrome, resulting in changes in neuromotor, autonomic and cognitive-behavioural functions (Sternbach, 2003). The 5-HT Syndrome is generally believed to be a drug-induced condition, primarily caused from the use of 5-HT increasing drugs which result in a 5-HT excess (Gillman, 1999). Research has found that the release of 5-HT also mediates the psychomotor stimulant effect caused by MDMA use, resulting in increased levels of energy (Fletcher et al, 2002).

While positive reinforcement and the role of the 'reward' pathway have been discussed above, negative reinforcement also needs to be addressed. Once an individual is addicted to a drug it is negative reinforcement that is responsible for reinforcing the behaviours. When a user becomes dependant on a drug, it is likely that drug-seeking behaviours will become negatively reinforced. For example, a smoker may seek to smoke a cigarette in order to alleviate their anxiety and calm their nerves. Similarly, a heroin-dependant individual who has abstained from use may seek to alleviate the symptoms of withdrawal and resort to seeking out heroin.

To become addicted to a drug, users must first become tolerant to and then suffer withdrawal when refraining from using the substance. Tolerance is a state of progressively decreasing responsiveness to a drug's effect (Koob & Nestler, 1999). That is, increasingly larger doses need to be taken to achieve the same drug effect that a smaller dose originally caused (Carlson, 2007). For example, a chronic methamphetamine user will need to take larger and larger amounts of the drug for it to remain effective. It is believed that tolerance is caused by the body's attempt to compensate for the substances alterations to the brain (Camí & Farré, 2003). This is because when the effects of a drug alters systems in the brain over an

extended period of time, compensatory mechanisms work to produce the opposite effect to the drug. This helps to partially compensate for the 'disturbance' that is caused to the brain by the substance; however it often results in a larger dosage needing to be taken so the user can feel the desired drug effects (Carlson, 2007). When a user refrains from engaging in use of the drug, these compensatory mechanisms are often felt as withdrawal symptoms as they are unopposed by the original action of the drug of which they were compensating.

Withdrawal symptoms occur once a drug has been abstained from suddenly. The severity of symptoms and abstinence period needed to suffer withdrawal varies between drugs. It has been suggested that withdrawal symptoms of stimulant drugs are a result of low DA levels which have arisen from the brain's compensation for the effects of the substance (Camí & Farré, 2003). Often withdrawal symptoms are opposite to the initial drug effects (Carlson, 2007). For example, a user of heroin may feel euphoria and relaxation but on withdrawal of the drug feels dysphoria and agitation. Unfortunately, if withdrawal symptoms are inescapable, relapse is possible. It has been found that relapses of drug abuse involve the activation of the mesolimbic system of dopaminergic neurons (Carlson, 2007). Specifically, Robinson and Berridge (2003) suggest that activation of the mesolimbic dopaminergic system by an addictive drug gives 'incentive salience' to present stimuli. This means that the stimuli present at the time of drug-taking becomes associated with the drug behaviour. If during withdrawal, or a period of abstinence, the user then thinks about the stimuli they then feel an impulse to take the drug. These impulses in combination with withdrawal symptoms make it extremely difficult for a chronic user to maintain abstinence of the drug, and ultimately can lead to relapse.

In summary, substance abuse and dependence are complex conditions which require understanding of the underlying mechanisms. An individual who experiments with an addictive substance is at risk of becoming dependant if they continue use. Consequentially once a drug has been taken, positive reinforcement has potentially been activated via the DA

pathway and possibly the 5-HT pathway, which can ultimately direct the individual to a SUD. Once an addiction has been established, negative reinforcement via tolerance and withdrawal maintains drug use. Unfortunately as long-term negative effects are caused by drug use during adolescence, it is vital that all substances at risk of being taken by young people are tested thoroughly.

1.3 Prevalence of Drug Use in New Zealand

Worryingly, substance abuse and polydrug use is becoming more common around the world, including within New Zealand (Ministry of Health, 2010; Stansfield & Kirstein, 2005; Quintero, 2009). This is further evidenced by a Ministry of Health 2010 survey which found that nearly one in two (49%) New Zealander's had tried an illegal drug at least once in their lifetime (Ministry of Health, 2010). Among these, one in three individual's had first used drugs when aged 15-17 (34.3%) and 26.8% had first used drugs between 18-20 years (Ministry of Health, 2010). Additionally, research found that males in the 18-24 year age group used more drugs than females in the same age group, with 38.1% having participated in substance use compared to 29.8% respectively, during the previous 12-months. Comparatively, 22.3% of males compared to 27.9% females had used an illegal drug in the 16-17 year age group (Ministry of Health, 2010). These findings suggest that in New Zealand, females are using illegal drugs earlier in adolescence than males but in later adolescence, males are using drugs more than females. This supports why research investigating drug effects of exposure to substances during both early and later adolescence in males and females is needed.

1.4 Adolescence

Adolescence is a developmental period defined as the gradual transition from childhood to adulthood (Spear, 2000). While its definition does not pinpoint exact ages of adolescence, it should be noted that the definition generally extends to and encompasses puberty (Spear, 2000). During puberty, young people attain an adult-size body and become capable of producing offspring (Berk, 2006) yet their mindset may not mature simultaneously. During adolescence, the brain is undergoing many complex changes that can exert long-term influences on decision making and cognitive processes (Stainsfield & Keirsten, 2005). Choices are made by individuals during this period that can ultimately affect their adult identities as choices can impact upon adult functioning and even future adjustment (Spear, 2000). It has been found that adolescence is the developmental period in which most drug experimentation is undertaken, as more risky behaviours are engaged in (Arnett, 1996). Evidence suggests that an adolescent drug user is more likely to become dependent in adulthood (Gilvarry & McArdle, 2007).

1.4.1 Sensation Seeking – risk taking behaviours

During adolescence, there is a significant increase in risk-taking behaviours, including experimentation with alcohol and drugs, which can evolve to substance abuse or addiction (Conner et al, 2010; MacPherson et al, 2010; Stansfield & Kierstein, 2005). This has led to the concept known as ‘Sensation Seeking’. Sensation seeking has been defined as the personality trait which is characterised by the degree of an individual’s desire for novelty and intensity of sensory stimulation (Arnett, 1994; Arnett, 1996). The repercussion of this is that adolescents who are high in sensation seeking are willing to engage in reckless behaviours to obtain feelings of intense and/or novel sensation (Arnett, 1992). In addition, many studies have found that adolescence is a developmental period in which, sensation seeking appears to

be at its peak (Arnett, 1996; MacPherson et al, 2010). Reckless behaviours which have been found to be correlated with sensation-seeking include; driving at high speeds, alcohol use, sexual activity and importantly, drug use (Arnett, 1992; Arnett, 1996; Conner et al, 2010). Therefore, increased sensation seeking may be a developmental trait which can ultimately lead from adolescent drug experimentation to adult substance abuse or addiction. Research has found that the onset of risky behaviours is correlated with poorer health and emotional outcomes in later life (Machperson et al, 2010).

The ingestion of illegal drugs can be referred to as reckless, or as described above, sensation seeking where the reward is a novel sensation often felt as the ‘high’ or ‘rush’ from obtaining and/or using the drug. The Ministry of Health analysed data to determine rates of drug use among adolescents and adults in New Zealand in 2008. This data revealed that individuals in the adolescent (age 16-17) or early adulthood (age 18-24) age groups had participated in more drug use than adults older than 25 years (Ministry of Health, 2010).

While research has examined what forms of sensation-seeking adolescents undertake, many studies lack reasoning for the under-lying motivation to indulge in reckless behaviours. Conner et al (2010) completed a study in which the underlying mechanisms of sensation seeking were examined – that is, whether there is a genetic pre-disposition for sensation-seeking or whether it is environmentally influenced. Results found that in females, sensation-seeking appeared to be environmentally shaped whereas in males, there appeared to be a hypodopaminergic genetic risk. This means that only males appeared to be at genetic risk for the number of drugs tried by participants. Interestingly, this suggests that males and females may have different underlying triggers for sensation-seeking in the form of drug taking. The New Zealand Ministry of Health (2010) found that users of cannabis, amphetamines, cocaine, LSD, and prescribed stimulants were mostly adolescents and early adults. Sex differences were also found (Ministry of Health, 2010). This when combined with the theory of sensation

seeking, may begin to form a plausible model for determining the differences between sexes initiating drug use.

In summary, adolescence is a developmental period where risk taking and sensation seeking behaviours are increased. While multiple behaviours have been identified as sensation seeking, one of the most popular is drug taking. There are predispositions such as genetic and environmental influences which can predispose an adolescent and make them more likely to use drugs. Studies using twin participants' have found support for genetic factors having a greater influence on adolescent drug use than environmental factors.

1.5 Neurodevelopment

As described above, adolescence is a period of physical, cognitive and behavioural maturation between childhood and adulthood when the brain is undergoing complex and dramatic changes (Blakemore et al, 2010). During this period, nearly every tissue in the brain is undergoing change (Luciana, 2010). Early adolescence generally occurs around the onset of puberty when the individual is undergoing sexual maturation (Spear, 2000). Adulthood generally begins when the individual has attained a stable adult role and most pubertal transitions have ceased (Blakemore et al, 2010). While these two developmental phases occur simultaneously, they are not synonymous, as puberty is a transitional stage within adolescence (Spear, 2002). The brain is affected by pubertal hormones and this should not be disregarded when examining adolescent neurodevelopment. Neurodevelopment is a complex and intricate phenomenon which begins prenatally. From birth to adolescence, the brain increases in volume four-fold (Johnson, 2009) and within this time frame, essential chemical and structural changes are occurring constantly.

Neurodevelopment is critical during early adolescence as the brain is undergoing a vital maturation process (Laviola et al, 1999). There are changes in the brain's neurochemistry

and neuroendocrine control (Smith, 2003). Cellular changes such as increased myelination and synaptic pruning also occur (Romer, 2010). During periadolescence there is a great increase in the volume of synapses and receptors (Anderson, 2003). Synaptic pruning then serves to rid the brain of excess synapses which have not formed strong connections (Anderson & Navalta, 2004). It is estimated that as many as 40% of synapses in the brain are lost during adolescence (Anderson & Navalta, 2004). This is thought to begin at approximately 11 years of age, when the prefrontal cortex and parietal lobes begin the prolonged task of synaptic pruning which results in a thinning of cortical grey matter, it is at this time when myelination appears to increase (Romer, 2010). During this phase, the brain adopts more functional synaptic networks therefore becoming more efficient (Luciana, 2010). Additionally, the brain experiences maturation in specific anatomical structures, particularly the nucleus accumbens, amygdala, hippocampus and prefrontal cortex (Carlson, 2007). Each of these serves a separate, specific function. Specifically, the nucleus accumbens is involved with reward, motivation and addiction (Carlson, 2007; Nieuhaus et al, 2009). The amygdala regulates emotional reactions while the hippocampus is responsible for the formation of new memories (Carlson, 2007; Spear, 2000) and the prefrontal cortex is involved with making judgements and abstract reasoning (Carlson, 2007). It has been reported in a number of studies that the total amount of grey-matter in the brain also decreases after adolescence, but sharply increases prior to puberty (Luciana, 2010).

As previously discussed, many drugs exert their actions via the DA 'reward' pathway. This pathway involves the nucleus accumbens and prefrontal cortex- both of which undergo crucial maturation during adolescence. Additionally, interference with the brain's normal development can lead to region-specific deficits (Anderson & Navalta, 2004). This means that drugs taken during adolescence could ultimately affect the developmental outcomes for the individual during adulthood.

1.5.1 Neuronal Imprinting – long term effects of stimulants

Neuronal Imprinting is defined by Anderson and Navalta (2004) as the phenomenon whereby drug effects outlast the actual drug exposure itself. That is, drug effects appear to incubate. This notion has been evidenced in previous research by Malanga et al (2009) where prenatally drug-treated animals did not exhibit drug effects immediately after birth, but when retested during adulthood showed significant differences from non-treated controls. That is, the drug-exposed animals demonstrated different behavioural patterns and cognitive abilities to controls. Likewise, retrospective studies such as that of Bandstra et al (2010) found differences between prenatally cocaine/opioid exposed and unexposed children at three years of age.

Drug exposure during adolescence alters brain development in the brain region where the drug is active (Anderson & Navalta, 2004). This is proposed to be caused by the ‘normal developmental trajectory’ of the drug-affected brain circuit being altered in such a way that it differs from what would be predicted if the drug exposure had occurred in adulthood (Anderson & Navalta, 2004). While there are adaptive processes which the brain employs to adapt to these changes during adolescent drug exposure, often the effects caused are more extensive and permanent when compared to adult use of a drug (Anderson, 2003). As discussed previously, adolescence is a developmental period when the brain is undergoing major changes, including the pruning of synapses in order to reduce unnecessary connections. It is during this process that drug exposure will most likely have its greatest impact on brain regions undergoing active development compared to those that have reached their adult status (Anderson & Navalta, 2004; Anderson 2003; Lidow et al, 2001; Stansfield & Kierstein, 2003). Therefore, it has been proposed that chronic drug effects are incorporated in adolescents by integrating drug-induced changes into permanent developmental adjustment

(Anderson & Navalta, 2004); this unfortunately often results in an altered development trajectory for the drug-exposed individual.

Studies examining both adolescent and adult mammals tend to suggest that the younger, adolescent brain is more sensitive to the lasting effects of substances than adult brains (Smith, 2003). Fone et al (2002), found evidence that two low doses of MDMA twice daily, for three consecutive days in adolescent rats caused long-term changes in social interaction in adulthood. It was found that 12 – 29 days after the last MDMA injection, that the treated rat's social interaction was significantly decreased (41%) compared to controls. Aarons et al (1999) researched adolescent drug and alcohol use in humans. Their findings suggest that even limited substance use during adolescence may be associated with adult depression, feelings of a lack of sense of purpose and lower self-esteem. Perhaps the most significant research for drugs affecting later life may be that involving Methylphenidate (MPH), or Ritalin, as it is commonly known. As use of MPH is increasing and children as young as two are being diagnosed with Attention Deficit Hyperactivity Disorder (ADHD), it is important to know the outcomes of chronic use.

Over long-term use, MPH can trigger a persistent alteration of monoaminergic transmission which if occurring during development, can potentially have cytoarchitectural and neurochemical consequences later in life (Gray et al, 2007). Monoamines are important regulators of brain development and altering them during development may affect myelination, synaptogenesis and gliogenesis processes (Levitt et al, 1997). Carlezon et al (2003) found that adolescent exposure to MPH can lead to the development of depression like behaviours in adulthood. Studies such as those of Brandon et al (2001) and Anderson et al (2002) also indicate that exposure to MPH in adolescence can influence drug behaviours in adulthood. Given that previous studies have indicated that adolescent exposure to drugs can result in life-long changes to brain-circuits and thus development, there is good reason to

believe that the use of methyldone during adolescence may also affect behavioural development. Therefore, any differences observed in adulthood behaviours would be consistent with neuronal imprinting theory.

1.6 Anxiety and Assessing Anxiety like Behaviours in Rats

Anxiety is an aversive emotional and motivational state which occurs in a perceived threatening environment (Eysenck et al, 2007). The development of anxiety disorders has been linked to drug use (Ludwig et al, 2008; Piper, 2007). The phenomenon of anxiety is a naturally occurring sensation in humans and is conceptualised by Power and Dalglish (1997) as the following:

“A state in which an individual is unable to instigate a clear pattern of behaviour to remove or alter the event/object/interpretation that is threatening an existing goal” (Power & Dalglish, 1997, pp. 206–207).

While excessive amounts of anxiety can be debilitating, small amounts can often be performance enhancing (Andrews et al, 2003). In addition, it should be noted that when severe anxiety occurs in the right conditions, it is thought to be occurring at a normal level. Anxiety symptoms include; heart palpitations, muscle tension, difficulty in breathing, hyperventilation and trembling (APA, 2000). When anxiety occurs at disproportionate levels, is chronic and/or occurs when no ‘risk’ is evident, this is symptomatic of an anxiety disorder (Millan, 2003).

Anxiety disorders are common in modern society. Research has found support that polydrug use of illicit substances is correlated with the development of anxiety disorders later in life, even after a period of abstinence. Sareen et al (2006) found chronic use of cocaine, stimulants, hallucinogens and heroin was significantly associated with the development of anxiety disorders. Of these, the highest association was between panic disorder and

hallucinogen use (Sareen et al, 2006). Providing further support is a more recent study by Bedi et al (2010) which found MDMA and cannabis polydrug users reported more anxiety symptoms than did users of legal drugs. In addition, their symptoms were of a higher severity. It is critical that the etiology and treatment of anxiety and its related disorders is understood so as to enable the right treatments to be devised for the patient in question as it is often the use of drugs and underlying causes which leads to a person developing an anxiety disorder. For example, Scott et al (2010) found that while use of MDMA did correlate with mood disorders, neither lifetime nor recent use of MDMA was correlated with the severity of anxiety symptoms. However, evidence was found that environmental factors including, lifetime trauma, recently occurring stressful events, tobacco and recent polydrug use, did significantly predict the severity of anxiety symptoms felt by the MDMA user.

Clinical studies and animal studies are the most commonly used techniques for researching anxiety. Anxiety is thought to be an exclusively human trait and at best can only be modelled in animals, not reproduced (Martin, 1998). However, good animal models still provide heuristic and predictive value as the psychopharmacological profile observed in the model can provide a base for estimation of clinical activity (Martin, 1998). For a model to be useful and therefore reliable, it should consist of three features. These are as follows; an animal anxiety model should reproduce behavioural and pathological features, allow investigation of the neurobiological mechanisms and allow reliable evaluation of anxiolytics features (Martin, 1998). As stated above, anxiety has been described as a subjective state and therefore, an exclusively human trait. Consequentially it is up to the human researcher to deduce what the animal is feeling. This is done by assessing the behaviours which the animal is exhibiting to infer the emotions they might be experiencing. However, in order for this to be achieved it is important that the researcher is aware of the differences between fear and anxiety when using animal models.

Understandably, an animal's anxiety-like behaviours are different to a human's and researchers have identified animal behaviours which are thought to encompass the required anxiety state. For example, common anxiolytic behaviours recorded in rats are increased defecation; decreased grooming; decreased exploratory behaviours such as rearing; increased freezing and longer emergence latencies. In addition, specific symptoms of various human anxiety disorders have animal equivalent behavioural symptoms (Cryan & Holmes, 2005). For example, in agoraphobia where a human symptom may be avoidance of places from which escape could be difficult, a rat may exhibit increased avoidance of exposed well-lit areas. For social anxiety, a human symptom may be anxiety provoked by social situations which leads to avoidance behaviours; a rat may display low social interactions with unfamiliar rats. A human diagnosed with Generalised Anxiety Disorder (GAD) may experience difficulty in concentrating; a rat may exhibit impaired sustained attention. For a full table of such examples refer to Cryan and Holmes (2005). It is important to note that while many human symptoms are able to be modelled in animals, others are not; such as the feeling of loss of control during a panic attack (Cryan & Holmes; 2005), it is therefore vital that animal researchers are aware of which behaviours exhibited are able to be analysed and referred to as 'anxiety-like'.

1.7 Ecstasy (MDMA)

The recreational use of amphetamines is common across many cultures (Clemens et al, 2004; Clemens et al, 2007) especially in social situations such as dance clubs (Cassel et al, 2005). Often MDMA is taken with a cocktail of other drugs such as ethanol, cocaine, amphetamines and cannabis presumably to enhance cognitive effects; however, these combinations can be lethal (Cassel et al, 2005). MDMA use has increased in recent years among adolescents in the United States of America (Bull, et al, 2004; Martins et al, 2008) and it has been reported as being one of the four most commonly used illicit drugs in the world

(Young et al, 2005). In New Zealand, use has also increased and a survey by the New Zealand Drug Society showed that users were finding MDMA more accessible (Wilkins & Sweetser, 2005). Worryingly, individuals in both early and late adolescence are engaging in use of MDMA. This is further evidenced by the Ministry of Health's recent survey which found that overall, 3.7% of New Zealanders had tried MDMA at least once. Of this, 13.9% of users had first tried MDMA when they were younger than 18 years, and 29.5% had first tried between ages 18-20 years (Ministry of Health, 2007; Ministry of Health, 2010). Sex differences were also evident in substance use patterns. Specifically for MDMA, in the 16-17 year age group during the previous twelve months 3.3% of males had used MDMA compared to 1.4% of females. This percentage rose in the 18-24 year age group where 8.9% of males compared to 4.9% females had used MDMA in the twelve months prior to the research being undertaken (Ministry of Health, 2010)

MDMA acts as a CNS stimulant resulting in feelings of euphoria and pleasant effects such as enhanced well-being, increased energy levels and sensuality (Young et al, 2005). Negative effects in human users include induced hyperthermia, hallucinations and paranoia (Wilkins & Sweetser, 2005). Even short term use can lead to 5-HT Syndrome, enhanced impulsivity, anxiety, memory deficits and behavioural changes (Adori et al, 2010; Cassel et al, 2005; Piper, 2007; Meyer et al, 2004; Noller, 2009; Reveron et al, 2010; Reneman et al, 2000). Morley et al (2001) treated male rats with a high or medium dose of MDMA over two consecutive days. When tested 12-weeks later, both groups exhibited greater anxiety-like behaviours than control rats. In addition, those rats treated with a high dosage of MDMA showed impaired memory compared to those treated with a medium dose and controls. Fone et al (2002) found further support that use of MDMA during adolescence affects social interaction in adulthood. Male adolescent rats were treated with either 7.5mg/kg of MDMA twice daily for three consecutive days or a saline vehicle. Analysis found that in adulthood, social interaction was reduced in rats treated with MDMA compared to controls.

Understanding the underlying mechanisms of MDMA is vital when looking at methylone due to the chemical similarities between the two drugs. MDMA is a potent releaser and/or inhibitor of presynaptic 5-HT, DA and norephedrine which stimulate the CNS often resulting in a strong desire to socialise and hallucinatory experiences for the user (Shima et al, 2009). MDMA increases the monoamine neurotransmitter concentrations in the synaptic cleft of the brain. This then results in two separate mechanisms; first, drug inhibition of the plasma membrane transporter and second, drug evoked release of monoamine transmitters (Cozzi et al, 1999). Research has shown that long-term exposure to MDMA results in 5-HT depletion in the cortex, hippocampus and striatum (Cassel et al, 2005; Fone et al, 2002; Reneman et al, 2000). Chronic exposure can result in severe 5-HT toxicity, also referred to as the 5-HT Syndrome, which is caused by an excess of 5-HT release (Piper, 2007). Symptoms consist of confusion, hallucinations, coma, shivering, diarrhoea and seizures- if left untreated, 5-HT toxicity can be lethal (Piper, 2007; Clemens et al, 2007). MDMA produces long-term damage to 5-HT neurons and it has been found by studies that use often increases anxiety levels in both human and animal studies (Piper, 2007). Clemens et al (2007) found evidence that moderate to high doses of MDMA administered to rats' resulted in long-term social anxiety. In addition, after a single high dose, it was found that MDMA resulted in persistent changes in brain neurochemicals as well as behaviours in rats.

Numerous behavioural changes in both animals and humans after MDMA exposure have been observed in various studies. Research by Bhattachary and Powell (2001) supported previous evidence that MDMA use caused impairments in memory. The authors found a deficit in verbal, but not visual memory, by testing a selection of human non-users, novice users, regular-users and heavy users. Of the MDMA user groups, all displayed poorer verbal fluency and delayed prose than non-users. A review by Piper (2007) compared MDMA users to controls to examine the long-term responses to MDMA during periadolescence, adolescence and adulthood; this was achieved by reviewing recent literature using both human and animal

participants. Long-term behaviour found to be affected in adolescent users included learning, memory and anxiety levels. Major depressive disorders were just as likely to occur prior to as after MDMA use but Dysthymia Disorder often developed only after use. In addition, adolescents and early adult users were more likely to experience a variety of anxiety problems including GAD, phobias and panic attacks than adult users. For an extensive analysis of the review, refer to Piper (2007).

A compelling study by Meyer et al (2008) suggests that dosing rats in a regimented, brief, consecutive cycle may not be adequate to mimic adolescent use. To rectify this, the authors' developed a rat model to reproduce a human adolescent's intermittent MDMA use to simulate many features of recreational weekend use of MDMA. Results showed that intermittent use caused deficits in object-recognition memory and increased impulsivity later in life. Reduced sensitivity to a 5-HT agonist challenge was also noted. Perhaps most interestingly, results showed that the rats SERT-immunoreactive fibre density was significantly reduced in the hippocampus but not in the neocortex. The authors suggest that during adolescence, the hippocampus may be particularly vulnerable to moderate MDMA exposure. Data to date provide evidence for long term alterations in neurochemical and behavioural aspects of MDMA users, this further confirming that such exposure can have negative behavioural outcomes in adulthood.

1.8 Methylone

Methylone is a relatively new 'party drug' which first made its appearance in the Netherlands in 2004 (Bossong et al, 2005) but is now gaining popularity in Japan, Europe and the U.S.A (Shima et al, 2009). The drug has been likened to Ecstasy (MDMA) due to the two drugs' behavioural and chemical similarities (Bossong et al, 2005; Cozzi et al, 1999).

However, effects for users of the drugs are not identical with methylone being reported as almost having the “same effectiveness of MDMA, but it does not produce the same effects. It has an almost anti-depressant action, pleasant and positive, but not the unique magic of MDMA” (Bossong et al, 2005, pg 322). Methylone’s worldwide street name is ‘Explosion’ but in New Zealand it is also commonly referred to as ‘Ease’ (www.methylone.com, accessed March 2nd 2010).

Methylone was originally used as an anti-depressant and an anti-Parkinson agent in 1996 (Jacob & Shulgin, 1996). More recently, in 2004, methylone was used as a party drug in the Netherlands and Japan and is often referred to as an ‘ecstasy-like substance’ (Bossong et al, 2005). This new designer drug was often sold in liquid form via the internet, and when it first appeared in the Netherlands and Japan, it came in a small tube with the label stating: “Room Odorizer Vanilla. Do not ingest” and “Keep away from children. Never consume more than one bottle”. Despite these warnings, users report they ingest the liquid in order to reach the desired psychoactive effect the drug causes (Bossong et al, 2005). Due to a lack of data and literature, it is unclear if methylone is taken primarily in liquid or pill form by users in New Zealand.

In New Zealand, the legality of methylone is at best, unclear. For example, ‘Ease’ was briefly available as a legal party pill and safe alternative to MDMA in 2006. Six months after sales began; the drug was pulled from shops, trials by the importing company terminated and all available pills seized by the police. This was due to findings that methylone is similar in its chemical structure to MDMA (Armstrong, 2006). Although there is no specific reference in the ‘New Zealand Misuse of Drugs Act 1975’ to methylone being illegal in New Zealand, because of its similarities to MDMA, it is considered to be a Class C illegal drug. Minutes taken at a 2006 Ministry of Health Expert Advisory Meeting of Drugs Committee Meeting reveal that methylone is currently classed as a ‘Class C7’ drug until drug trials begin (Experts

in Drugs Advisory Committee, 2006a). At a later meeting in November, 2006, the committee meeting minutes' state that the chairman is not satisfied that Class C7 is a suitable classification for methylone (Expert in Drugs Advisory Committee, 2006b). It is unclear at the time of writing whether the proposed trials have commenced.

Methylone is chemically related to MDMA although little is currently known about its toxicity, metabolism and pharmacological properties (Kamata et al, 2006). The stimulatory effects of both drugs are most likely due to their ability to increase catecholamines and 5-HT (Shimizu et al, 2006). Like MDMA, methylone also acts on monoaminergic systems in the brain (Cozzi et al, 1999; Bossong et al, 2005) but unlike MDMA, it has a weak effect in the vesicular monoamine transporter (Shimizu et al, 2006). Methylone is a benzylic ketone analogue of MDMA (Bossong et al, 2005), and contains an additional ketone oxygen atom at the benzylic position of the molecule (Cozzi et al, 1999). Although similarities are evident, Cozzi et al. (1999) found that methylone is threefold less potent than MDMA at inhibiting 5-HT uptake into platelets. This may result in less of a 'rush' for the user of methylone than of MDMA. The similarity in the two drugs' chemical structure can be seen in Figure 1 below.

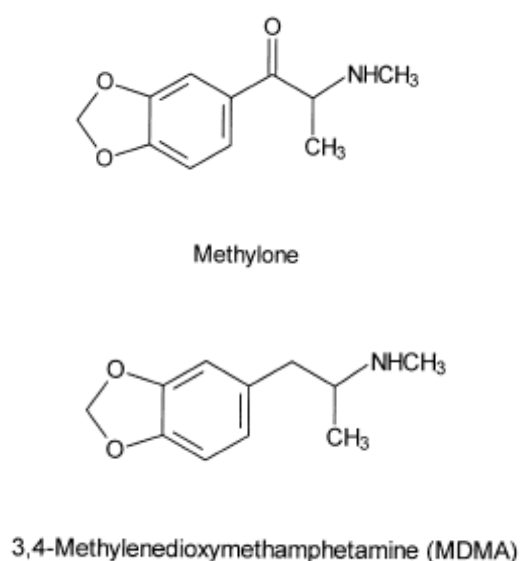


Figure 1. The Chemical Structures of MDMA and Methylone

Cozzi et al, (1999) stated methylone is less effective than MDMA at inhibiting platelet 5-HT accumulation but is as effective as MDMA in its inhibitory effects on the DA and noradrenaline transporters. Due to methylone's similarity in structure to MDMA, it is thought that health risks arising from using methylone would be similar to that of MDMA (Kamata et al, 2006). There is however, a lack of available research confirming this. It was found by Dal-Carson et al, (1997) that methylone could substitute for MDMA in rats trained to discriminate between MDMA and a saline solution. Although methylone may be recognised as a 'safe alternative' to MDMA, this is an unfounded depiction due to the lack of data documenting long-term effects in human adolescent users. If the hypothesis of the current study is correct, it is expected that differences will be found between saline and methylone treated rats.

To date, research into the long-term effects of taking methylone during adolescence is sparse. To illustrate the current lack of literature, when beginning research for this thesis, only a total of four journal articles could be located which mentioned methylone in the research databases. This is alarming due to some adolescents' possible extensive use of methylone during the critical brain development period. Even more alarming is that none of these studies were aimed at adolescent users. Therefore, it is vital to ascertain whether the use of methylone in adolescence affects brain/behavioural development, and therefore behaviour in adulthood.

1.9 Current Study

The current study investigated long term behavioural effects of either early or late adolescent exposure to methylone. Rats being treated during early adolescence began receiving injections of a methylone and saline mix on Post Natal Day (PND) 35, those beginning treatment in late adolescence began on PND 45. Both groups received ten consecutive days of drug exposure and were then tested at PND 90 in various behavioural

tests. The importance of such a study on methylone is vital as evidenced by the lack of current literature available on this drug. Valid and reliable data is required to gain valuable knowledge about the behavioural and developmental implications when using this drug during adolescence. Therefore, much more research needs to be undertaken on methylone to evaluate its health risks to users.

2.0 Aims and Hypotheses

The current study was conducted to gain valuable knowledge about the long-term behavioural effects arising from methylone exposure during adolescence. As literature to date lacks research aimed at adolescent exposure, the main aim of this study was to investigate possible outcome differences between methylone-exposed and saline-treated rats. In addition, it was aimed to determine whether use of methylone during either early or late adolescence had a differing impact on behavioural development in adulthood. This was achieved by using two different adolescent aged groups of rats, treating them for ten days with either methylone or a saline mix and then comparing their behaviours in adulthood. Lastly, it was intended to identify whether there are any sex differences in the behavioural development of methylone exposed rats.

Due to a lack of research being available on adolescent methylone users, specific predictions were not possible for this study. However, as there are chemical similarities between methylone and MDMA, it was thought that differences between treatment and control groups might be found in terms of emotionality and anxiolytic behaviours. This is due to previous studies finding behavioural differences between MDMA exposed and control rats (Young et al., 2005; Mehan et al, 2002; Maldonado & Novaro, 2000).

It was hoped this research will improve current knowledge about the long term effects of exposure to methylone. As no literature was able to be sourced which examined whether exposure to methylone during adolescence would affect developmental trajectories and therefore adult behaviours, it was thought this research would make a valuable contribution to current data.

3.0 Method

3.1 Subjects

The subjects were 80 PVG Hooded rats from the breeding colony in the Psychology Department, University of Canterbury, New Zealand. Of these, 40 were females and 40 males. All rats were housed in the Animal Facility within the Psychology Department in a humidity-controlled ($48\% \pm 10\%$) and temperature regulated ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) environment. The rats maintained a constant light-dark cycle of 12 hours light, 12 hours dark and had free access to food and water. On PND 30, the rats were weaned and housed in plastic cages with a measurement of 475-mm x 280-mm x 320-mm. The animals were separated into small groups (3-4) of the same sex for the duration of the experiment. All procedures were approved by the University of Canterbury Animal Ethics Committee (See Appendix A).

On PND 34, the rats were randomly divided into two separate experimental groups, each consisting of 20 females and 20 males, with a total of 40 animals. The first group ($n = 40$) began the drug treatment phase on PND 35, when the rats were entering the human developmental period equivalent to early adolescence. The second group ($n = 40$) began treatment on PND 45 (late adolescence). Within each group, the rats were randomly assigned into smaller same-sex groups of ten and received either a methylone or a saline solution for ten consecutive days. All doses were administered at approximately 1030 hours during the light phase of the animals' light-dark cycle. It was decided to test from both PND 35 and PND 45 in order to encompass the earlier and later stages of adolescent neurodevelopment phases in rats (Smith, 2003).

On PND 90, when the animals were entering the human-age equivalent of adulthood, all rats were behaviourally tested on measures of anxiety, curiosity, habituation and activity level. By testing in adulthood, it allowed for comparison of data to determine whether chronic exposure to Methylone in either the early adolescent or late adolescent developmental stages

resulted in long-term behavioural consequences in adulthood. If such behavioural differences were found between the experimental and control rats, it could be assumed that these differences were ascribable to chronic methylone use. By comparing both early and late adolescence it would allow for determination of whether or not the current developmental stage the brain is in during drug use results in distinctive behavioural differences in adulthood compared to controls.

3.2 Drug Use and Rationale for Dosage

2-methylamino-1-(3,4-methylenedioxyphenyl)propane-1-one (methylone) was synthesised by and purchased from BDH Synthesis, Lower Hutt, New Zealand. Methylone was mixed with 0.9% saline to produce doses of 8mg/kg; this was then administered to the rats in a volume of 1ml/kg. During either early or late adolescence, all rats were exposed to either a chronic methylone or saline solution for ten consecutive days. The two groups beginning treatment in early adolescence began treatment on PND 35. The 'PND 35 methylone' group was administered methylone at a dose of 8mg/kg, whilst the 'PND 35 control' group received a 1mg/kg dose of saline daily. Both groups received intraperitoneal injections (i.p.) from PND 35 to PND 44. The remaining two groups began treatment on PND 45. The 'PND 45 methylone' group received methylone solution at a dose of 8mg/kg while the 'PND 45 control' group was administered 1mg/kg saline solution. Both groups received i.p injections daily from PND 45 to PND 54. Body weights were recorded daily for each rat, and injection volumes were adjusted accordingly to maintain the dose level. Although research suggests users prefer to ingest methylone orally in a liquid or powder form (Bossong et al, 2005; Shimizu et al, 2007), injections were chosen as the drug vehicle due to ease and accuracy of delivery. It was also important to maintain accuracy and consistency when administering the drug because of the small quantities being administered to each animal

therefore, i.p. injection was determined the best way to ensure these requirements were achieved effectively. The rats were injected at approximately 1030 hours each day.

Pilot observations in the University of Canterbury animal laboratory indicated that 8mg/kg of methylone was behaviourally effective to rats without being toxic. This was thought to be a moderate dosage which would ensure that the rats treated with methylone would not suffer a fatal overdose. Pilot studies were necessary because of the lack of scientific literature relating to effective doses for rats.

Table 1. *Days of Exposure with Methylone (mg/kg) or Saline (S)*

Group	Days of Methylone (MTH) Treatment	1	2	3	4	5	6	7	8	9	10	Total Methylone (MTH) Exposure
PND35 Control (Male,10)	0	S	S	S	S	S	S	S	S	S	S	0mg/kg
PND35 Control (Female, 10)	0	S	S	S	S	S	S	S	S	S	S	0mg/kg
PND35 Methylone (Male,10)	10	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	80mg/kg
PND35 Methylone (Female, 10)	10	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	80mg/kg
PND45 Control (Male,10)	0	S	S	S	S	S	S	S	S	S	S	0mg/kg
PND45 Control (Female, 10)	0	S	S	S	S	S	S	S	S	S	S	0mg/kg
PND45 Methylone (Male,10)	10	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	80mg/kg
PND45 Methylone (Female, 10)	10	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	MTH (8)	80mg/kg

3.3 Apparatus and Behavioural Measures

The current study utilised four empirically studied and supported tests for measuring anxiety-like behaviours in rats. These were; the open-field test, the light-dark-box test, responsiveness to brightness change in a Y-maze and the acoustic startle test. It was decided to employ these tests as they were simple to use and no training was required of the animals. Most importantly, each also avoided unnecessary invasive physical harm or food deprivation. Testing began once each animal had reached approximately PND 90, when it could be regarded as the rats having attained adulthood (Anderson, 2003).

Each test elicits behaviours in a rat which are generally caused by a novel situation; these are then recorded and used as an index of anxiety. Different behaviours are provoked in each of the different tests. Typically in the open-field, a more anxious animal will spend more time in the corners of the apparatus and less time in the exposed centre (Hall, 1934a). They will also rear and groom themselves less (Prut & Belzung, 2002). In addition to these behaviours, a more anxious animal will often defecate more than a less anxious animal (Hall, 1934b; Wills et al, 1983) therefore, all faecal boluses were counted during the trial. In the light-dark test, an animal experiencing higher anxiety will take a longer time to enter the light side of the box than a less anxious animal (Hascoët et al, 2000). In a Y-maze, a more anxious rat will enter the novel arm less and thus spend a lesser amount of time in it than a less anxious rat (Aitchison & Hughes, 2006). Finally, in the acoustic startle test, a more anxious rat will jump higher and more frequently in response to a sudden noise (Koch, 1998).

The experimental room in which all tests were conducted maintained a stable temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a humidity control $48\% \pm 10\%$ and dim lighting of 44 lux. All tests were conducted between 1000 and 1600 hours, in the light phase of the rats' normal light/dark cycle. Tests were completed over four consecutive days. With the exception of the second day of testing, when rats completed two tests, each animal completed one test per day. To guarantee ample rest between tests on day two, it was ensured that there was a minimum

of one hour between them. Over the total four-day testing period, each rat completed the open-field twice, the light-dark preference box once, the Y-maze once and the acoustic startle test once. To avoid order effects, it was randomly decided in which order individual rats would complete the tests.

3.3.1 Open Field

The open field elicits anxiety by separating the animal from its home cage and social group and putting it into an inescapable novel environment (Prut & Belzung, 2002; Walsh & Cummins, 1976). Originally developed by Hall (1934a) to be a test of emotionality in rats, it is now one of the most popular tests used in animal studies of anxiety (Prut & Belzung, 2002). The open-field is also commonly used to assess the behaviourally stimulant and sedative properties of drugs (Gould et al, 2009).

Each rat was placed in the centre of a Perspex open field, and its location and behaviours were recorded every three seconds for a total duration of five minutes. The open field consisted of measurements of 600 x 600 x 305-mm high and the apparatus was placed on a 700-mm high table. The walls were constructed from clear Perspex which allowed for easy observation of each subject's behaviour. The floor consisted of black Perspex divided into a numbered 4 x 4 grid of 16 equal sized (150 x 150-mm) squares. Four squares were in the centre and twelve occupied the periphery of the field. Dim (44 lux) fluorescent lighting illuminated the apparatus 1,225-mm overhead.

The recorded behaviours consisted of location in the box, rearing up or leaning on hind legs and grooming. For the purposes of this study, grooming was defined as the touching of hands-mouth; hands-ears; mouth-sides; mouth-tail; mouth-feet; mouth-genitals; mouth-abdomen; feet-head and feet-sides (Moyaho et al, 1995; Pleskacheva, 1995). The number of times the rat was located in a different square from where it was three seconds

previously provided locomotor activity (ambulation) scores. In such a situation, it has been found that rats generally prefer the outside squares to the centre ones (Prut et al, 2000), therefore frequency of total corner and centre occupancy was calculated. Faecal boluses were also counted as defecation in rats increases when stressed (Hall, 1934a).

This test and these observations are designed to measure general activity and emotional reactivity (Hall, 1934a; Royce, 1977; Walsh and Cummins, 1976). The open field was completed by each animal twice, once on the first day of testing and then again, approximately 24-hours later. This was done to determine whether anxiety was decreased during the rat's second time in the field. Any differences could then be assumed to have been caused by memory of the previous entry into the open-field. It has been previously shown by both Toshioka (1932) and Hall (1934b) that the frequency of faecal boluses and urination decreases with each subsequent trial until practically all faeces and urine excretion is eliminated. It was therefore the postulation that if a difference was found between groups, that it could be attributable to methyllone.

3.3.2 Light/Dark Preference Box

The light-dark test is centred upon rats' innate avoidance of brightly illuminated areas (Hascoët & Bourin, 2009; Sanchez, 1996). Each rat is placed in the dark compartment of the light-dark box for 30-seconds, with the guillotine slide down separating the compartments. The slide is then withdrawn and the latency of entering the light side is recorded. Once the rat has entered the light-side, it is allowed free access to both sides for five minutes with the total time spent in the light side, and the entries of it, being recorded. If a rat did not enter the light side after five minutes, the trial was terminated and a latency of 300-seconds recorded. A computer program was used to record the rats' entries and calculate the length of time spent in each side. After each rat had entered the box, it was cleaned down and disinfected with 20%

Paraquat Blue. This test is designed to measure emotional reactivity, as it involves a conflict between a fear of brightness and the tendency to explore a novel environment. Higher emotionality, or anxiety, is indicated by the tendency to avoid the light side (Hughes et al, 2004; Hascoët & Bourin, 2009).

The light-dark box measured 700 x 150 x 200-mm. In the centre, a partition created two sides; one was black painted Perspex (the dark-side) and consisted of measurements of 200 x 150 x 200-mm. The other side was white painted Perspex, consisting of measurements of 500 x 400 x 200-mm. A sliding door was in the centre of the partition which was lifted to allow the access to both sides. An animal was determined to have fully emerged when four paws had appeared from the dark-side, and it was then when the five minutes would begin.

3.3.3 Responsiveness to Brightness Change in the Y-maze

In the responsiveness to brightness change test, each rat was placed in the stem of a Y-maze, with one arm containing a black insert, and the other a white insert. After a six-minute acquisition trial, the rat was removed and placed in a black compartment at the base of the apparatus. The maze was then wiped down and disinfected with 20% Paraquat Blue and the white and black inserts replaced by two clean black ones. The rat was then placed back into the stem for a three-minute retention trial. During this trial, the total number of entries of each arm was recorded as were the total time spent in each arm. These records enabled subsequent calculation of (a) the percentage of entries of the novel arm, (b) the percentage of time spent in the novel arm, (c) the total entries of both arms, and (d) the total time spent in both arms was. During the acquisition trial, half of the rats (n= 40) entered the maze with the black insert on the left side, while the remaining half began the trial with the black insert on the right. It was randomly determined which side the black insert would be on for each rat. Within each testing group, five rats completed the test with the insert on the left, and five

completed it with the insert on the right. This test is designed to measure novelty preference as well as short-term memory and activity (Hughes, 2001; Young et al, 2005). Research by Young et al (2006) found that in a double-Y maze, neither a low-dose nor medium-dose of MDMA affected rats' memories in the task. Due to the chemical similarities between methylone and MDMA, it was expected that comparable results would be found using methylone in a single Y maze.

3.3.4 Acoustic Startle

Startle has been defined by Koch (1998), as the fast response to sudden or intense stimuli which most likely innately protects the organism from either a predator or injury caused by a blow to the body. An animal's unlearned acoustic startle response is evident as soon as their hearing is functional; this has been found to be approximately PND 12 in rats (Koch, 1998). In the acoustic startle test, a loud sound is emitted every 30-seconds to measure a rat's startle reaction and it is thought by Koch and Friauf (1995) that an acoustic startle response may be primarily influenced by an animal's innate fight/flight tendency. A hypothesised acoustic startle pathway is displayed below; this has been adapted from Koch, 1998.

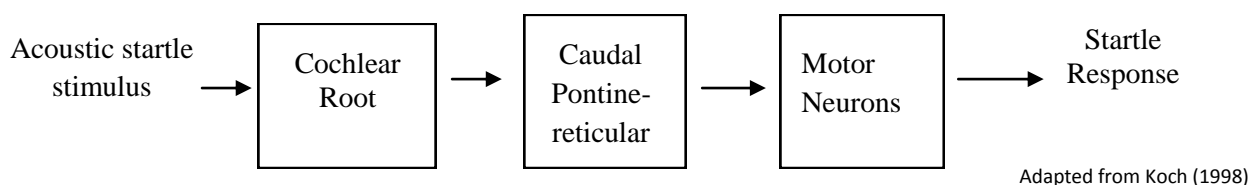


Figure 2: Hypothesised Acoustic Startle Pathway

The acoustic startle apparatus entailed a set of four separate chambers. Each chamber housed one rat for the duration of each trial. The chambers were constructed from sound-attenuating melamine to minimise sound carry, and each measured 66-mm x 300-mm x 560-mm. Within the chamber, a speaker and holding cage were held by stainless steel rods and the rat was placed in the inescapable holding cage. Acoustic startle was elicited in the animals via white noise bursts emitted by a programmable audio generator every 30-seconds. The noise intensity was 95-dB and it lasted 100-ms. In order to measure the startle amplitude of each animal, the holding cage was mounted on a load cell-based startle platform measuring 250-mm x 115-mm x 45-mm. This platform allowed for movement elicited by the rat after a noise burst to be recorded by Med Associates software as 'startle amplitude'.

Each rat was placed inside a startle chamber and completed an acquisition block (block one) to enable them to become familiar with their surroundings. The first block consisted of sound bursts emanating every 30-seconds for ten trials. The second block then immediately began and a sound burst was emitted every 30-seconds for 20 trials. The average startle movement amplitude in response to each sound burst comprised each animal's startle score. This was determined by the amount of movement of the startle platform.

The acoustic startle response is characterised by rapid contractions of both the face and skeletal body following a loud, unexpected sound (Koch & Friauf, 1995). This can appear as a 'jump' when an animal is startled. A platform on the base of the box in which the rat was enclosed measured the frequency of how often the rat jumped when startled. A computer programme was used to calculate the startle amplitude of each animal. It is accepted that a more anxious rat will jump both more frequently and higher than a less anxious rat (Rosen & Schulkin, 1998).

4.0 Statistical Analysis:

The main focus of this study was to examine adult behavioural development outcomes following chronic exposure to methylene during either early or late adolescence. The expectation was that the animals exposed to methylene during early or late adolescence would demonstrate different behaviours during testing than controls. All data were subjected to ANOVAs to assess the effects of adolescent exposure to methylene (treatment condition), age when treated (treatment age) and sex of the rats. Open-field and acoustic-startle data were also examined via repeated measures ANOVAs for differences between the two testing days and the two testing blocks respectively. In addition, declines between the two testing days in open-field ambulation and rearing were calculated (and expressed as percentages decline) in order to assess possible effects of the experimental conditions on between-days habituation of these responses. Such habituation can be viewed as an elementary form of learning and memory (Thiel et al, 1998). (Note: negative values in this habituation measure indicated that the response increased rather than decreased between the two testing days.) Sex differences were included in the analyses due to sex differences in brain maturation during adolescence as there are brain differences in maturation rates between sexes (Anderson, 2003). It is likely that exposure to methylene may affect each sex differently and therefore, lead to different long term outcomes between sexes.

5.0 Results:

5.1 Open-field Results

Each animal was tested twice in the open field, with each trial approximately 24-hours apart. The results for the main effects of drug condition, treatment age, sex and testing day are outlined in Table 2.

Table 2. Mean (\pm S.E.M) open-field responses for each treatment condition, treatment age, sex and testing day, and results of ANOVAs.

Treatment condition	Saline	Methylone	F(1,72)	P
Ambulation	56.92 (1.24)	52.24 (1.18)	6.42	<0.015
Rearing	30.49 (1.18)	30.11 (1.48)	0.18	>0.6
Centre occupancy	10.99 (0.78)	11.65 (0.86)	0.04	>0.8
Corners occupancy ^a	46.46 (0.89)	46.09 (1.35)	0.09	>0.7
Grooming	2.69 (0.35)	2.34 (0.21)	0.78	>0.3
Faecal boluses ^a	0.97 (0.25)	2.00 (0.27)	7.30	<0.01
Days 1-2 % ambulation decline	-2.17 (3.90)	-6.73 (5.19)	1.19	>0.2
Days 1-2 % rearing decline	-21.97 (7.33)	-8.76 (6.50)	1.10	>0.2
Treatment age	PND35-44	PND45-54	F(1,72)	P
Ambulation ^c	53.59 (1.22)	55.10 (1.31)	0.37	>0.5
Rearing ^c	29.84 (1.57)	30.73 (1.14)	0.13	>0.7
Centre occupancy	11.27 (0.92)	11.43 (0.73)	0.11	>0.7
Corners occupancy ^a	44.20 (1.08)	48.31 (1.21)	5.43	<0.025
Grooming	2.74 (0.29)	2.26 (0.27)	1.72	>0.1
Faecal boluses ^{ab}	2.03 (0.30)	1.05 (0.24)	6.51	<0.02
Days 1-2 % ambulation decline	2.35 (3.35)	-11.71 (5.61)	4.00	<0.05
Days 1-2 % rearing decline	2.26 (5.04)	-31.67 (7.53)	11.75	<0.001
Sex	Females	Males	F(1,72)	P
Ambulation	55.91 (1.25)	52.78 (1.24)	2.35	>0.1
Rearing	34.54 (1.35)	26.02 (1.01)	22.27	<0.001
Centre occupancy	9.6 (0.69)	13.10 (0.87)	8.32	<0.005
Corners occupancy	49.31 (1.01)	43.20 (1.16)	13.72	<0.001
Grooming	2.96 (0.34)	2.04 (0.18)	5.13	<0.03
Faecal boluses ^b	1.29 (0.27)	1.79 (0.28)	1.28	>0.2
Days 1-2 % ambulation decline	-10.82 (4.64)	1.46 (4.66)	3.00	>0.08
Days 1-2 % rearing decline	-12.99 (5.43)	-16.42 (8.21)	0.30	>0.5
Testing day	Day 1	Day 2	F(1,72)	P
Ambulation	53.96 (1.10)	54.72 (1.19)	0.48	>0.4
Rearing	29.02 (1.01)	31.54 (1.21)	6.56	<0.015
Centre occupancy	13.12 (0.75)	9.57 (0.67)	17.88	<0.001
Corners occupancy	44.45 (1.01)	48.06 (1.13)	7.08	<0.01
Grooming	2.84 (0.31)	2.16 (0.19)	5.46	<0.025
Faecal boluses	1.96 (0.27)	1.11 (0.20)	10.09	<0.005

^aTreatment condition x treatment age interaction significant (see text and Figure 4)

^bTreatment condition x sex interaction significant (see text and Figure 5)

^cTreatment age by testing day significant (see text and Figure 3)

5.1.2 Ambulation

Ambulation scores were significantly affected by the drug treatment. Saline-treated rats moved across more squares in the open field than methylone-treated rats. No other main effect was significant.

5.1.3 Rearing

All rats engaged in the same amount of rearing regardless of treatment age or treatment condition. However, females reared significantly more often than males. There was a significant interaction between treatment condition and testing day ($F(1,72) = 10.03$, $p < 0.005$) as outlined in Figure 3. This reveals that rats exposed to methylone from PND 45 reared themselves significantly more. The same effect was not found in rats beginning methylone treatment on PND 35, or rats treated with saline.

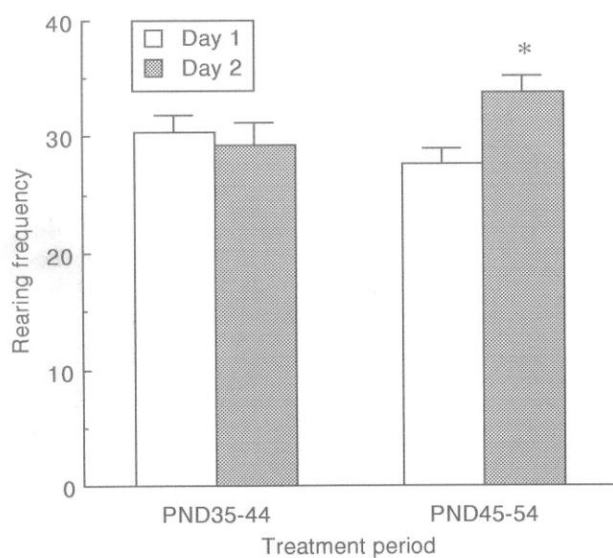


Figure 3: Mean (\pm SEM) rearing frequencies on Day One and Day Two of open-field testing for each treatment age group.

5.1.4 Centre Occupancy

Centre occupancy was significantly affected only by sex and testing day. Males occupied the centre of the open field significantly more often than females, and for both sexes combined, significantly less of this behaviour occurred on the second testing day than on the first.

5.1.5 Corners Occupancy

Although the main treatment effect for occupancy of corners was not significant, a significant treatment condition x treatment age interaction ($F(1,72) = 8.46$, $p < 0.005$) outlined in Figure 4 revealed that rats treated with methylone from PND 45 (but not from PND 35) occupied the corners significantly more often than those treated with saline.

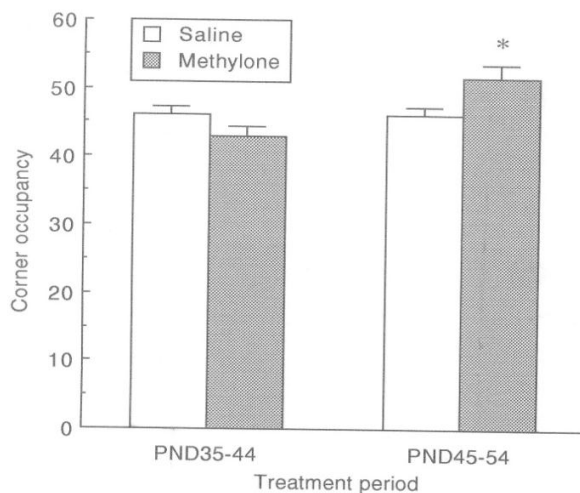


Figure 4: Mean (\pm SEM) frequencies of corner occupancy for saline- and methylone-treated rats administered the drug during each treatment period.

5.1.6 Grooming

While female rats groomed themselves significantly more often than males, this response was not affected by treatment condition or treatment age. However, significantly less grooming was evident on the second testing day than on the first.

5.1.7 Faecal Boluses

Although numbers of faecal boluses were significantly increased by methylone treatment, as shown by a significant treatment condition x treatment age interaction ($F(1,72) = 9.00, p < 0.005$), this effect only typified rats treated from PND 35 (see Figure 5).

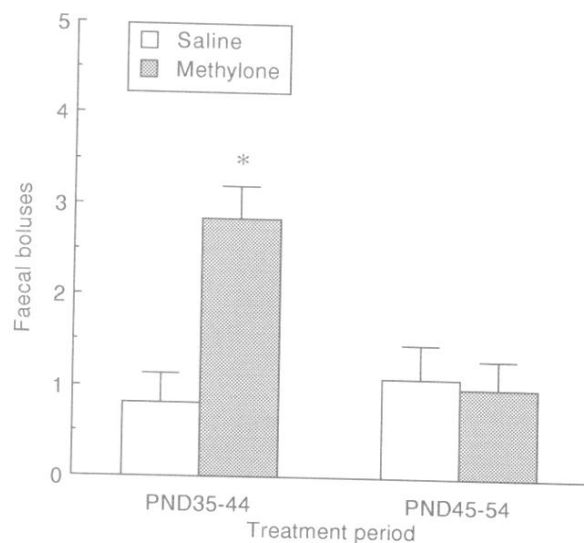


Figure 5: Mean (\pm SEM) numbers of faecal boluses for saline- and methylone-treated rats administered the drug during each treatment period.

A significant treatment condition x sex interaction ($F(1,72) = 11.24, p < 0.002$) outlined in Figure 6 revealed that the methylone effect was only significant for female rats.

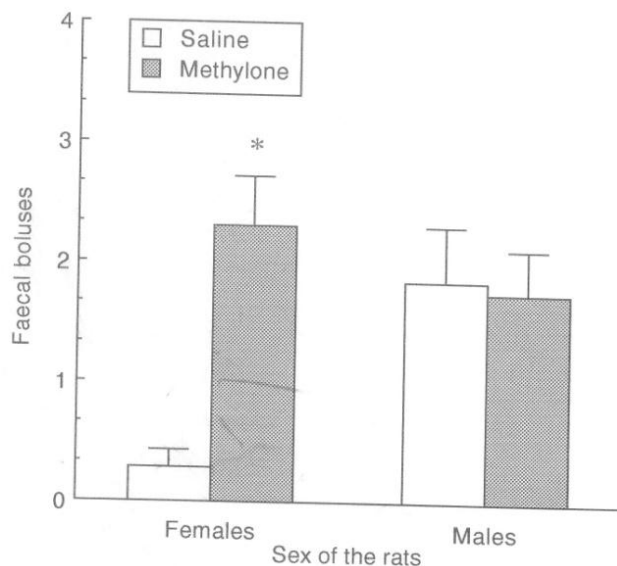


Figure 6: Mean (\pm SEM) numbers of faecal boluses for female and male rats treated with saline or saline- and methylone.

5.1.8 Between-days Habituation of Ambulation and Rearing

Percent decline between days one and two in ambulation and rearing was significantly affected by treatment age. In both cases, the response decreased between the two testing days for rats treated from PND 35, but increased for those treated from PND 45. The declines were not affected by either methylone treatment or sex. If methylone effects had been found, this may have signified effects on elementary learning or memory.

5.2 Light/Dark Box Results

In the light-dark box emergence test, each animal was tested once in adulthood on PND 90. ANOVAS were applied to the data to assess any methylone effects, the adolescent period of treatment (PND 35 vs. PND 45) and sex differences, as well as interactions between any of these. Table 3 below depicts main effects for the two measures analysed; i.e. number of transitions and time in light side.

Table 3: Mean (\pm S.E.M) responses in the light-dark box for each treatment condition, treatment age and sex, and results of ANOVAs

Treatment condition	Saline	Methylone	F(1,72)	P
Transitions	8.49 (0.75)	7.49 (0.55)	1.15	>0.2
Time in light side	67.43 (5.39)	65.63 (6.83)	0.04	>0.8
Treatment age	PND35-44	PND45-54	F(1,72)	P
Transitions	7.77 (0.59)	8.19 (0.73)	0.09	>0.7
Time in light side	56.22 (5.31)	77.36 (6.57)	5.65	<0.025
Sex	Females	Males	F(1,72)	P
Transitions	9.15 (0.67)	6.73 (0.58)	6.96	<0.015
Time in light side	70.86 (6.09)	61.93 (6.21)	0.87	>0.3

As shown in the previous table, there was no significant treatment effects for either number of transitions or time spent in the light side. The rats treated in late adolescence (PND 45) spent longer in the light side than the rats treated during early adolescence (PND 35). Overall, females made more transitions between the light and dark sides of the box than males.

5.3 Responsiveness to Brightness Change in a Y-maze Results

Each rat was tested once in the Y-maze at PND 90. Analyses were conducted via ANOVAS to assess the effects of the drug treatment, the adolescent period of treatment, sex and interactions between any of these. The measures analysed were entries into both arms, time in both arms, percentage of entries into the novel arm and percentage of time spent in the novel arm. The results are presented in Table 4.

Table 4: Mean (\pm S.E.M) responses in the Y maze for each treatment condition, treatment age and sex, and results of ANOVAs.

Treatment condition	Saline	Methylone	F(1,72)	P
Entries of both arms	6.15 (0.47)	5.25 (0.37)	2.30	>0.1
Time in both arms	75.51 (5.33)	74.16 (5.47)	0.03	>0.8
% entries of novel arm ^a	59.31 (3.20)	51.88 (2.61)	3.31	>0.07

Treatment age	PND35-44	PND45-54	F(1,72)	P
Entries of both arms	6.12 (0.45)	5.28 (0.40)	2.05	>0.1
Time in both arms	78.14 (5.85)	71.53 (4.87)	0.74	>0.3
% entries of novel arm ^a	56.83 (2.69)	54.35 (3.24)	0.37	>0.5
% time in novel arm	56.28 (3.62)	52.50 (3.72)	0.54	>0.4

Sex	Females	Males	F(1,72)	P
Entries of both arms	6.30 (0.40)	5.10 (0.44)	4.09	<0.05
Time in both arms	81.21 (4.57)	68.46 (5.96)	2.76	>0.1
% entries of novel arm	56.54 (2.03)	54.64 (3.69)	0.22	>0.6
% time in novel arm	53.97 (3.19)	54.81 (4.11)	0.03	>0.8

^aTreatment condition x treatment age interaction significant (see text and Figure 6)

5.3.1 % Entries of Novel Arm

There were no significant main effects of treatment, treatment age or sex for the percentage of entries into the novel arm. However, a significant interaction between treatment condition and treatment age ($F(1,72) = 6.16$, $p < 0.02$) showed that rats treated from PND 35 (but not PND 45) with methylone entered the arm significantly less often than those treated with saline (see Figure 7).

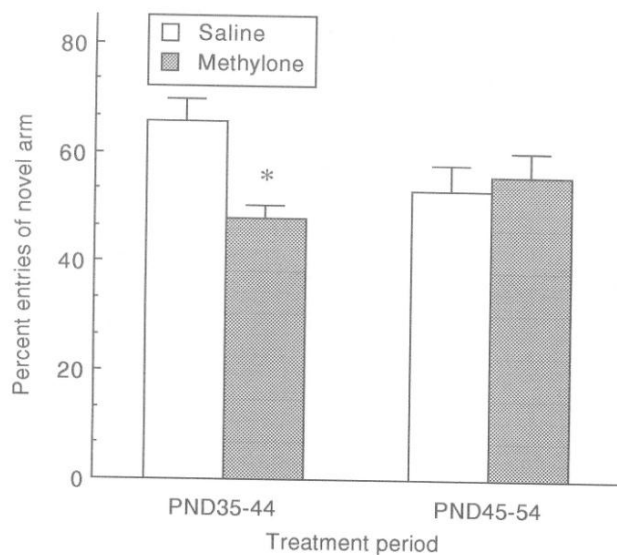


Figure 7: Mean (\pm SEM) percentage of entries into the novel arm for rats treated with saline or methylone during both treatment periods.

5.3.2 % Time in Novel Arm

Methylone-treated rats spent significantly less time in the novel arm than control rats. Control rats spent over half (65.62%) their time in the novel time whereas methylone-treated rats spent under half (47.62%). No sex differences were found between groups. There were no significant treatment age or sex effects for this measure.

5.3.3 Entries of Both Arms

There were no significant treatment condition or treatment age effects for the total number of entries of both arms, but females made significantly more entries than males.

5.3.4 Time in Both Arms

There were no significant main effects or interactions for the time spent in both arms.

5.4 Acoustic Startle Results

Results are presented in Table 5. Analyses of both blocks of the trial were included as this allowed for the determination of whether differences between treatment groups had also occurred in block one when all rats were first exposed to the new environment. Similarly, it enabled the analysis of whether any group differences had occurred during the acquisition period (block one) and if they had, whether they were continued in block two, or vice versa.

Table 5: Mean (\pm S.E.M) startle amplitudes in the acoustic startle apparatus for each treatment condition and age, testing block and sex, and results of ANOVAs.

Treatment condition	Saline	Methylone	<i>F</i> (1,72)	<i>P</i>
Startle amplitude ^d	421.32 (34.21)	369.26 (27.59)	2.67	>0.1
Treatment age	PND35-44	PND45-54	<i>F</i> (1,72)	<i>P</i>
Startle amplitude	421.35 (30.71)	369.22 (32.29)	2.68	>0.1
Testing blocks	Block 1	Block 2	<i>F</i> (1,72)	<i>P</i>
Startle amplitude ^d	435.52 (24.07)	357.80 (24.05)	18.92	<0.0001
Sex	Females	Males	<i>F</i> (1,72)	<i>P</i>
Startle amplitude	302.30 (19.64)	481.59 (33.41)	21.37	<0.0001

^dTreatment condition x testing blocks interaction significant (see text and figure 8)

As revealed in Figure 8 below, during block one of the trials, no significant differences between groups were observed. Although both the testing blocks and sex main effects were significant, this did not characterise either the treatment condition or treatment age effect. A significant treatment condition x testing blocks interaction ($F(1,68) = 5.33$, $p < 0.025$) outlined in Figure 8 showed that methylone treatment led to smaller startle responses than saline

treatment in block 2 but not in the block 1 trial. Female rats also exhibited lower startle amplitudes than males for both blocks combined.

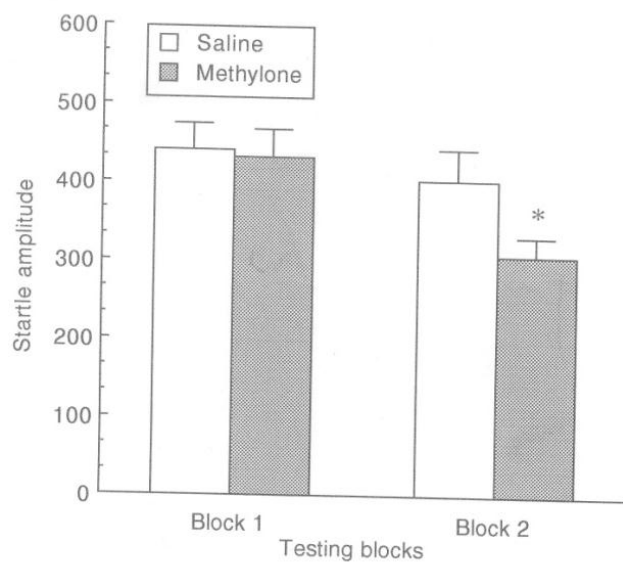


Figure 8: Mean (\pm SEM) startle amplitude during testing blocks 1 and 2 for saline- and methylone-treated rats.

6.0 Discussion of Results

In this study, 40 rats were split into age groups consisting of early and late adolescence and either treated with a vehicle of methylone or saline. All rats were exposed to ten consecutive days of treatment where they were administered either 8mg/kg methylone or a saline solution via i.p. injections. The early-adolescent rats began treatment on PND 35 while those being treated in late adolescence began treatment on PND 45. Following the final tenth injection, the rats received no further handling other than that needed necessary for cleaning of their home cages. Once the animals had reached adulthood on PND 90 (Anderson, 2003), the rats began behavioural testing. Different treatment age groups were included for this study to allow for comparative data to be gathered. Interpretations could then be made from any significant results about the behavioural adult outcomes of using methylone during two different critical brain maturation periods.

6.1 Summary of Results

Results revealed differences in behaviours displayed between the control and treatment groups. That is, it appears rats exposed to methylone during adolescence display more emotionality than control rats in aspects of the measured behaviours. The following summary of results supports this and has been tabulated in Table 6 for easy reference.

Table 6: *Observed Behavioural Effects Following Chronic Methylone Exposure.*

Apparatus and measure	Behavioural Effects of Chronic Methylone Exposure Compared to Control's Observed Behaviours.
Open field:	
Rearing	no effect
Ambulation	<i>decreased in both PND 35 and 45 groups</i>
Occupancy of centre squares	no effect
Occupancy of corners	<i>increased but only in PND 45 group</i>
Grooming	no effect
Faecal boluses	<i>increased but only in PND 35 group.</i>
Light-dark box:	
Time in the light side	no effect
Transitions	no effect
Y maze:	
% time spent in the novel arm	<i>decreased in both PND 35 and 45 groups</i>
% entries of the novel arm	<i>decreased but only in PND 35 group</i>
Entries of both arms	no effect
Time in both arms	no effect
Acoustic Startle :	
Startle amplitude	<i>decreased in block 2, no effect in block</i>

Note. Where 'no effect' is stated, no significant differences were found between controls and exposed animals.

In the open field, methylone-treated animals ambulated less than controls but defecated more. Significant interactions were found that further supported a methylone effect (see above text). Rats treated with methylone from PND 45 occupied the corners more than those treated with methylone from PND 35 or controls. In the Y-maze, methylone-treated animals spent less time in the novel-arm than controls. Rats treated from PND 35 with methylone made significantly less entries into the novel arm than did their control counterparts. This result was not found between the PND 45 treatment groups. An inconsistency in these results was observed in the acoustic startle test where methylone-treated animals startled significantly less in Block Two than controls did.

Increased defecation is regarded as a sign of increased anxiety (Hall, 1934b; Royce, 1977; Kontinen et al, 1999). Methylone-exposed animals defecated more in the open field, suggesting they were more anxious than controls. In addition, methylone-treated rats ambulated less than controls did. As low ambulation is indicative of higher anxiety, this result lends further support to the interpretation that the methylone-treated rats were displaying more emotionality in the open field. A further methylone effect was found in PND 45 exposed animals in the open field as this group occupied more corners of the apparatus than did those exposed to methylone from PND 35 or controls. This is further discussed below. High occupancy of the corners of the apparatus indicates higher anxiety as the rat is avoiding open areas (Kontinen et al, 1999). Collectively, these results suggest that in the open field test, methylone-treated animals displayed higher anxiety.

The above findings are in contrast to results from acoustic startle. Studies have found that more anxious rats' will jump higher after a startle stimulus, thus giving a higher reading of 'startle amplitude' (Koch, 1998) In the startle test, statistical analysis revealed a significant interaction between startle amplitude, treatment condition and testing blocks suggesting that rats treated with methylone were less anxious in block two of the trials than controls. This is supported by the significant decrease of startle amplitude exhibited by methylone-treated rats in the second block. It was found that during the acquisition period (block 1), controls and methylone-treated rats were relatively equal in terms of their startle amplitude. This difference may reflect that methylone-exposed rats were exhibiting less anxiety-like behaviours in the second block than controls as they had become more accustomed to the 'white noise' being heard and were therefore, jumping less resulting in a lower startle amplitude.

In the Y-maze, methylone-treated groups spent a significantly lesser time in the novel arm than controls did. This suggests one of two things, firstly, that methylone-exposed rats were less curious about the novel arm because they were more anxious or secondly, that short

term memory was impaired by methylone. This possibility arises from the rats needing to remember the nature of both arms of the maze in order to successfully identify the one which has changed in the second trial of the test (Hughes, 2000; Hughes & Maginnity, 2006).

6.1.1 Treatment Age Differences and Methylone Interactions

A main effect in decrease of percentage rearing decline was identified. This demonstrated a significant difference between treatment age groups in Day 1-2 % rearing decline, where rats in the PND 35 group decreased but rats in the PND 45 group increased in rearing frequency between days one and two of trials (refer Table 2). This suggests that rats in the PND 35 group were more anxious on the second day of trials as lower rearing in rats is indicative of higher emotionality as less exploratory behaviours are being exhibited (Adamec et al, 1991; Katz et al, 1981; Anderson & Hughes, 2008).

While no main effect was found for rearing, an interaction was found between treatment ages, rearing frequency and day of trials (refer Figure 3). This interaction demonstrates that rats treated with methylone from PND 35 decreased in their rearing frequency from day one to day two of testing. In contrast, the methylone-exposed rats treated from PND 45 demonstrated an increase in rearing on their second day of testing. This suggests that rats treated from PND 45 may have been less anxious than those treated from PND 35 as evidenced by the increase in rearing. It is proposed that those treated from PND 45 may have been exhibiting less anxiety-like behaviours than PND 35 as demonstrated by the significant main effect found in percentage rearing decline. An alternative explanation for this vast difference between treatment age groups is that the rats treated from PND 35, exhibited less habituation behaviours and thus possibly demonstrated impairment in learning and/or memory. Therefore, those rats treated from PND 35 may have had difficulty in recalling the same open field apparatus from the previous days testing.

A main effect for treatment age and faecal boluses was also found as rats in the PND 35 group defecated more than those in the PND 45 group during open field trials. Increased defecation is thought to be an indication of high emotionality (Hall, 1934a) therefore; this finding appears to indicate that rats in the PND 45 group were less anxious than those in the PND 35 group as they defecated less. Furthermore, an interaction was found between treatment age, treatment condition and faecal boluses (refer to Figure 5). This indicates rats exposed to methylone from PND 35 defecated significantly more than those treated from PND 45, suggesting that those exposed to methylone during early adolescence, exhibited higher anxiety behaviours. Therefore, this interaction may account for the main effect found between treatment ages and faecal boluses.

A main effect was found for percentage ambulation decline and treatment age. Rats in the PND 35 group ambulated less on day two of trials than on day one. The opposite was found for rats in the PND 45 group, whose ambulation increased between trial days. This further supports the above findings in that rats in the PND 45 group may have been less anxious than those in the PND 35 group. No interaction with treatment condition was found, revealing that this result was not altered by a methylone effect.

For corner occupancy and treatment age in the open field, a main effect was identified (refer Table 2) where rats in the PND 35 occupied the corners significantly less than those in the PND 45 group. This may suggest that by not leaving the ‘safeness’ of the corners and entering the open space in the centre, rats in the PND 45 group were exhibiting higher anxiety-like behaviours than those rats in the PND 35 group (Gould et al, 2009). This may be partially explained by the significant treatment condition and treatment age interaction (refer to Figure 4) revealing rats treated with methylone from PND 45 occupied the corners significantly more than those treated from PND 35. This result is inconsistent with the above findings, as rats in the PND 35 group appear to be less anxious than those in PND 45 group in

this observation, therefore opposing results from the other analysed behaviours of the open field.

In the light-dark box, the rats treated in late adolescence with methylone spent a significantly longer time in the light side than did those treated in early adolescence. Previous studies suggest that more highly anxious rats tend to stay in the dark side of the box (Bourin & Hascët, 2003; Walsh & Cummins, 1976). By being in the light side, the rat is being more curious and exhibiting exploratory behaviours (Bourin & Hascoët, 2003; Hughes et al, 2004; Sanchez, 1996).

In summary, results suggest that in many of the behavioural measures, there was a significant difference between controls and the methylone treated groups. However, exposure to methylone during either early or late adolescence does not appear to result in vastly different behaviours between methylone-treated groups in the light-dark box, Y-maze and acoustic startle. While those exposed in late adolescence did appear to be more anxious in some of these behavioural measures, when all the behaviour measures are taken into account, these were not of a significant nature to conclude a vast difference between treatment age groups. The same cannot be stated for the open-field test where four main effects were found, of which two had a significant interaction with treatment condition.

6.1.2 Sex Differences

A number of sex differences were observed for each of the behavioural tests. In the open-field, females demonstrated significantly more rearing, corner occupancy and grooming than did males in the open field test whereas males occupied the centre more often. Females rearing significantly more than males is consistent with previous studies (Archer, 1975; Tavhibana, 2001) and many studies have indicated that lower levels of rearing are an index for higher anxiety (Aitchison & Hughes, 2006; Herbert & Hughes, 2009; Kontinen et al,

1999; Ludwig et al, 2008;). An interpretation of this may lead to the presumption that males were more anxious during testing than females. However, as males also occupied the centre of the open-field more than females, an indicator of lower anxiety (Prut et al, 2000), this leads to some confusion in interpreting results as these two behaviours are contradictory and place doubt on the connotation of anxiety-like behaviours in this test.

Defecation is indicative of high ‘emotionality’ in a rat and suggests the rat is anxious or in a stressful state (Hall, 1934a). A methylone effect was observed in the females’ faecal boluses scores where those treated with the drug defecated significantly more often than males. This result suggests that methylone-treated females displayed significantly more emotionality than their male counterparts. Interestingly, previous findings have shown that males defecate more often than females in the open field (Archer, 1975; Aitchison & Hughes, 2006). In the present study, it would appear that treatment with methylone during adolescence subsequently made females display more anxious behaviours than males. Additionally, irrespective of the nature of their adolescent experience, females groomed themselves significantly more often than males, a behaviour suggestive of higher anxiety (Komorowska & Pellis, 2004; Moyaho et al, 1995; Pleskacheva, 1995). This study found no significant sex differences were observed in ambulation scores.

In the light-dark box test, females spent a significantly longer period of time in the light side than males did. They also made more transitions between the light and dark sides although this difference was found to not be statistically significant. Rats that spend more time in the light side of the box are generally assumed to be less emotional or anxious (Hascoët & Bourin, 2009) thereby suggesting that females were displaying less anxiety. This result is consistent with Y-maze results where females made more entries into the arms and also spent a longer period of time in them (Hughes & Neeson, 2003), indicating that females generally exhibit more exploratory behaviour than males (Anderson, 2003; Fernandes et al,

1999). Both the Y-maze and light-dark box results support this view. Significant sex differences were found in acoustic startle with females showing lower startle amplitudes than males. Although this might suggest that the females were less anxious than males in the startle apparatus, the result was more likely due to their lighter body weights (Lehman et al, 1999).

7.0 General Discussion

Results supported the primary expectations of this research that differences in emotionality would be evident between methylone-exposed and control rats. It was found that rats exposed to methylone during adolescence displayed more anxiety-like behaviour than saline-treated rats. On further examination of the effects of age of methylone treatment (early versus late adolescence) on behavioural development, it did not appear being exposed during earlier adolescence resulted in any significant differences in adulthood than if exposure commenced in late adolescence.

7.1 Neuronal Imprinting

Changes in methylone-treated animals' anxiety-related behaviours were analysed to determine whether treatment had affected levels of subsequent adult anxiety. This was done by using empirically supported behavioural tests such as the open-field, the light-dark preference box, acoustic startle, and responsiveness to brightness change in a Y-maze. Such tests have been used to model human symptoms of anxiety-like behaviours in rats. Behaviours recorded and analysed included; rearing, defecation, ambulation, startle amplitudes and emergence latencies. Results revealed an increase in anxiety-like behaviour in rats treated with methylone. This supported the theory of neuronal imprinting as it is thought these differences could be directly attributable to the exposure to methylone during adolescence.

This research intended to find evidence of neuronal imprinting after adolescent exposure to methylone. Consequently, significant differences were found between methylone exposed and control animals and the aim of being able to conclude evidence of neuronal imprinting were achieved. That is, the effects of methylone on the adolescent developing

nervous system were observable even after a period of abstinence from the drug. Contrary to original expectations, few differences were found between the two different treatment-age groups in adult behaviour. As the brain is undergoing vital yet different maturation processes during these separate adolescent phases, it was expected that methylone exposure would have its greatest impact on the specific brain area undergoing more active development at the time of exposure resulting in differing developmental trajectories (Anderson & Nalvata, 2004). This would then be reflected in behavioural differences in adulthood between the treatment-age groups. Therefore, as the brain is experiencing different development stages during early and late adolescence, it was expected that different behaviours in adulthood would be observed between the two treatment age groups. In this study, this was generally not the case.

While it has been concluded that neuronal imprinting had occurred to an extent in methylone- exposed rats, it is unclear in which brain region this has originated in. As methylone is thought to increase concentrations of monoamine transmitters, including 5-HT, DA and noradrenalin within the synaptic cleft (Kamata et al, 2006), it is suggested that increased levels of these during adolescence resulted in a differing amount produced in adulthood. Additionally, as DA is implicated in the 'reward pathway', increased levels during adolescence may have resulted in differing activation of this structure in adulthood, possibly making the exposed rats display different behaviours from controls.

Support of the neuronal imprinting theory established by this research is important due to the increase of New Zealand adolescents' consumption of illicit drugs (Ministry of Health, 2010). In addition, adolescence is a developmental period often associated with an increase in 'risk taking behaviours' (Arnett, 1994; Arnett, 1996; Conner et al, 2010; Stansfield & Kierstein, 2005) and decisions made during this time, can ultimately affect adult behavioural functioning. Specifically, it has been suggested that adolescent drug use has been associated with later life mental health problems (Aarons et al, 1999; Carlezon & Konradi, 2004; Piper,

2007). Use during adolescence has also been correlated with cognitive impairments (Renenan et al, 2000), behavioural (Meyer et al, 2008) and social changes (Fone et al, 2002). It is vital that the long-term effects of drug use during adolescence are reliably researched; this is particularly pertinent to recently established drugs such as methylone, of which there is relatively little literature about.

Although few neuronal imprinting differences were found between treatment ages, this does not mean that they will never appear. As the rats were behaviourally tested in early adulthood (PND 90), a second testing during later adulthood may have revealed more differences between those groups exposed to methylone during early or late adolescence. Additionally, a higher dosage of methylone during adolescence may have resulted in more differences being found between groups in adulthood.

7.2 Methodological Limitations

The methodological strengths and limitations of this study need to be addressed to be able to assess the extent to which these results can be generalised. During this study, several limitations became apparent that deserve discussion. For example, methylone was administered via i.p. injections once daily. Although little literature is available on this relatively new drug, it has been reported that users in Japan and the Netherlands primarily take it orally in liquid form to gain its psychotropic effects (Bossong et al, 2005). In New Zealand, it was the active ingredient of a now illegal party-pill named 'Ease' (Armstrong, 2006) and therefore consumed orally in pill form. Due to a lack of data, it is unknown whether methylone is still ingested in New Zealand primarily in this form. As rats were treated daily for ten consecutive days with 24-hourly injections, the treatment regime would not simulate that of a recreational human user. To gain an optimal model, the rats would have voluntarily consumed methylone orally at various times so as to simulate human usage

(Carlezeon & Konradi, 2004). However, as it was unknown whether the animals would voluntarily consume methylone, it was decided that i.p. injections would be preferable. This was to ensure each animal received an accurate and viable dose. As all animals received methylone by exactly the same method, it is important to stress that the results can be conclusively attributed to the drug's effects rather than to some other variable.

The second limitation which soon became apparent was that the acute effects of methylone were not measured immediately after exposure. While casual observations were made, these were not supported by objective measurements which would have provided useful, additional information. For example, from the third day of treatment, the rats treated with methylone appeared to become less fearful after injection. This was especially observable in the males. Interestingly, it was also observed that, after treatment, the methylone-exposed animals separated themselves from one another by standing in separate corners of their home cage while controls continued to huddle together in a group. The methylone-treated rats also engaged in more active locomotive activity than controls following treatment, particularly more ambulation and rearing. From the ninth day of treatment, many methylone-treated rats became aggressive during treatment and attempted to bite the researcher several times. However, once the drug had taken effect, these rats' behaviours resembled those of their previous day namely, high locomotive activity and less fear. Unfortunately, it is not known if this increase in aggression was needle-related or symptoms of a possible drug-related withdrawal. These casual observations suggested that it would have been highly beneficial to measure the acute effects of methylone- particularly as so little is known about this drug.

Due to the lack of literature about the toxicity of methylone, brain analyses of the deceased animals would have been very beneficial in order to gain information about the neurochemical effects of methylone on specific brain regions. For example, methylone

increases the concentrations of DA, 5-HT and noradrenalin (Bossong et al, 2005; Cozzi et al, 1999; Kamata et al, 2006) and was consequently first synthesised as an anti-depressant. Because DA is implicated in depression in humans, it would have been interesting to examine DA receptors in methylone-exposed rats. Any neurochemical differences found between saline- and methylone-treated animals could indicate long term consequences from exposure to the drug.

The presence of the observer during the testing phase of the thesis may have altered the rats' behaviours in tasks. This is especially so in the open-field and light-dark preference box, where the observer had to sit in close proximity to the apparatus in order to reliably record each animal's behaviours. Unfortunately, it is possible that the researcher's presence may have altered the animal's behaviours during behavioural testing. To reduce the likelihood of this, the observer reduced her body movements in order to ensure the animal would not be startled during testing. It is acknowledged that the use of an external video recorder would have eliminated this potential problem. However, as the same observer and room were used in all tests, it was hoped familiarity would help limit this.

Lastly, as the animals in these study were administered a dose considered to be 'medium', it would have been beneficial to also include doses considered 'low' as well as 'high'. This would have allowed the long term behavioural effects of different doses to be compared, thus giving a more detailed idea of the long term consequences of taking methylone during adolescence. This would have been especially interesting due to the inclusion of two different treatment ages (early versus late adolescence).

7.3 Methodological Strengths

A major strength of this study was the fact that rats were used as subjects rather than humans. Rats reach adulthood approximately 90 days after birth (Anderson, 2003). If humans were used it would have taken decades for the consequences of early and late adolescent methylone exposure to be detected. By using rats, a quicker and more efficient study was completed. Using rats also had the advantage of ensuring that all subjects had the same experiences such as, nutrition, handling and no prior drug history which meant that the results could be attributed to methylone effects, and not environmental factors.

Examining chronic use of methylone during both early and late adolescence allowed the long term effects to manifest themselves in two separate, important brain development phases. This gave the potential to gather more robust data and to gain as much information as possible about its effects when taken during adolescence. This is important as the brains is undergoing phenomenal growth and maturation at both PND 35 and PND 45 but are at different phases of development. This allowed the researcher to determine whether use of methylone during early adolescence was more detrimental in terms of long term consequences than in later adolescence. This did not appear to be the case in this study, potentially due to neuronal plasticity, and is discussed further in the general discussion.

As methylone is regarded by users as a ‘safe alternative’ to MDMA it is probable it may be used in combination with other psychotropic drugs. This study provides a starting point for future research to examine the use of methylone and polydrug use. As psychotropic drugs are often taken in combination with other drugs (Quintero, 2009), human research results can often be compromised. Limitations can include variations in drug purity, contamination and polydrug use. In addition, assignment to conditions in humans is generally non-random. The present study guaranteed that each animal received the same dosage of methylone during the treatment phase. The animal model used in this thesis controls for the

history of each animal, ensuring that differences are not caused by additional external factors. Each animal's environmental experiences were similar as they were handled, treated, tested and housed in the same manner. Therefore, by using rats this study eliminated some of the limitations found in human drug research ensuring that drug differences were more likely to be a result of methylone effects rather than external factors.

Lastly, this study serves as a pioneering research for effects of methylone as no known studies were sourced and consequentially, little is known about its behavioural effects. This study did not intend to mimic exact human use, but to find evidence for any long term effects caused by consuming methylone during either early or late adolescence. As no previous research has been aimed at adolescent exposure to methylone, all results are to be considered preliminary and to provide a starting point for future research. Consideration of both the strengths and limitations of this study is hoped to assist such future research.

7.4 Implications

This is the first known study to assess the long-term consequences of early and late adolescent exposure to methylone. Evidence of neuronal imprinting was found in adulthood testing. Due to these findings, it could be concluded that methylone may affect the brain's development during this critical maturation period resulting in different behavioural outcomes in adulthood. Although some behavioural differences were identified between animals exposed to methylone at early adolescence and those during late adolescence this was not of a sufficient nature for the author to conclude overall treatment age differences. However, when the different age groups were combined into one methylone-exposed group these animals generally displayed more anxiety-like behaviours than controls.

Due to a lack of available data, this study is important for understanding methylone's effects. Recent publications report methylone's increasing popularity as a drug of abuse,

especially in Europe, Japan, the Netherlands and the United States of America (Kamata et al, 2006; Shima et al, 2009). It is vital that as much knowledge as possible is gained due to the risk that this drug may also gain popularity and therefore, ease of availability, in New Zealand in the near future. As methylone has been reported to be a less potent alternative to MDMA (Bossong et al, 2005; Cozzi et al, 1999; Kamata et al, 2006; Shima et al, 2009), young people may expose themselves to this drug thinking it is safer and healthier. However, as there is a lack of research on methylone this may not be the case in the long term. This study endeavoured to gather valuable data to determine the consequences of its use.

Future studies which address the limitations discussed earlier, are crucial for detecting long-term effects of methylone which may not have been identified in this study. As research on MDMA has found long-term consequences of use during adolescence, it is not indisputable that future methylone studies will find similar results due to the similarities between the two drugs. For example, it has been found that adolescents exposed to MDMA have a greater risk of developing mood disorders such as Dysthymia (Piper, 2007). There is also evidence of long term anxiety (Clemens et al, 2007; Piper, 2007); deficit of cognitive abilities, for example verbal memory (Bhattachary & Powell, 2001) and memory (Reneman et al, 2000). Therefore, due to the chemical compound similarities between the two drugs, it is possible that use of methylone during adolescence could impact negatively upon the quality of life in adulthood. This is concerning due to New Zealand's increase of psychotropic drug use in past years (Ministry of Health, 2010).

8.0 Future Directions

This study has added valuable knowledge to the current literature by researching a drug on which previously, little data has been gathered on. However, some limitations must be discussed in the context of future research that is desirable. Following methylone exposure, hyperactivity was observed and later, after prolonged exposure, aggressiveness was noted by the researcher but was not empirically measured. As no published studies have previously measured the acute reaction to methylone, this would have added valuable knowledge to the current study. Additional studies investigating these casual observations would be justified due to the lack of current data on methylone. As increased energy is typical of MDMA exposure (Baylen & Rosenberg, 2006; Cohen 1995; Piper, 2007), such research would be extremely valuable in determining whether psychotropic effects of methylone are actually similar to that of MDMA. In addition, as hyperfunctioning of DA is implicated in the hyperactivity often seen in ADHD (Holroyd et al, 2008; Sagvolden et al, 2005), this could account for some of the increased ambulation observed in the methylone-exposed rats. This is especially relevant as methylone has been reported to increase DA activity in the mesolimbic areas. Likewise, the aggressive behaviour observed in the rats following the seventh day of exposure may be a form of withdrawal where DA levels in the brain have lowered between treatments resulting in an aggressive state. This aggressive state may be opposite to the initial methylone-related feelings of euphoria or pleasure, suggesting symptoms of withdrawal. However, much further research would need to be undertaken to support these statements.

Due to the vast availability of literature stating that adolescence is a stage of increased vulnerability towards using drugs, more research needs to be specifically aimed at this developmental period. Worryingly, there is a lack of research investigating the long term effects of drug exposure during adolescence. This statement is particularly true for methylone, a relatively new drug of which little is known about. Therefore, as adolescence is

a developmental period in which drug experimentation is common (Derringer et al, 2007; Fowler et al, 2007; Spear, 2003; Young et al, 2006) and as the brain is undergoing differing maturation processes throughout this stage, particular focus on this age group is essential when testing psychotropic drugs. Consequentially, it is vital that researchers continue to study the effects of drug exposure in both early and late adolescence.

As there is lack of knowledge regarding methyldone, more research is required to ascertain the long-term effects of the drug. Future studies need to examine differing doses to determine the differing long-term effects of exposure to low, medium or high doses. Consequently, the neurochemical effects of these could be analysed to reveal specific brain area changes caused by exposure. Likewise, acute effects of methyldone should be examined and possibly compared in a study to those of MDMA to add to knowledge about similarities and differences between the drugs. Lastly, a study examining the adulthood behavioural changes after adolescent exposure could include mid and late adulthood so as to examine whether later effects of the drug are found to add to current knowledge.

9.0 Conclusion

Within New Zealand, substance use and abuse is increasing (Ministry of Health, 2010). Gathering data on newly available and potentially addictive or harmful substances will give both users' and health providers' valuable knowledge which may assist in decision-making regarding the use and/or treatment of illicit drugs. Consequently, as this is the first known study to examine methylone's long term effects, it is hoped that that these results will increase awareness of the possibility of neuronal imprinting if methylone is used during adolescence. This is important as education of young people is crucial for providing them with relevant information about the dangers of drug use. It is such data as this which is hoped to deter young people from consuming a drug.

There are two main limitations of this study. Firstly, different dosages were not administered to the animals. This would have been valuable in examining the different long-term effects occurring from low, medium and high dosages. Likewise, it might have resulted in differences between treatments age groups which were not evident from only a medium dosage. Secondly, neurochemical evidence lacked in this study. While it was hypothesized that the increase in observed anxiety behaviours may have been caused by methylone's increasing effect on DA, 5-HT and noradrenalin resulting in a differing functioning of these systems during adulthood, there is no evidential proof of this. Even so, the results of this study are very apparent. Rats exposed to methylone during adolescence show an increase in anxiety-like behaviours in adulthood compared to their untreated control counterparts.

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Appendix A

Animal Ethics Committee

Secretary

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AEC Ref: 2010/06R



7 May 2010

Jollee Daniel
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Jollee

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Developmental implications for adolescent methylene users"

Approval has been granted:

- (a) for the use of 80 rats
- (b) for your research project to be undertaken from 7 May 2010 to 31 May 2011. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (<https://intranet.canterbury.ac.nz/research/ethics.shtml>).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, whichever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

Associate Professor Jim Briskie
Chair
Animal Ethics Committee

cc Animal Ethics Committee